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STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

OFFICIAL ORGAN OF BIOLOGICAL STAIN COMMISSION

JANUARY 1951

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Contains first 16 pages of 5-year index

Published by
BIOTECH PUBLICATIONS, Geneva, N. Y.

Entered as second class matter at the Post Office, Geneva, N. Y.

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

ISSUED QUARTERLY

SUBSCRIPTION PRICE $4.50 PER YEAR (U. S. CURRENCY)

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Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which must be complete in itself and contain all the important facts brought out in the paper. When possible the abstract should summarize the new methods in such form that a reader can follow the directions without consulting the body of the paper.

All staining formulae must be definite and accurate. The batch or certification number of the stains actually used by the author in such formulae should be included for reference purposes. In case of uncertified stains, not designated by any batch number, one should give as much information as available, i. e. name of manufacturer, approximate date of purchase, etc.

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STAIN TECHNOLOGY

VOLUME 26 JANUARY, 1951 NUMBER 1

SYNTHESIS OF SILVER PROTEINATES FOR NEUROLOGICAL STAINING

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Received for publication March 3, 1950

ABSTRACT.—**Soluble derivatives of the $AgNO_3$ precipitates of various split protein products were prepared by dissolving the precipitate in a 30–40% aqueous solution of pharmaceutical peptone (Cudahy). The split proteins used included pepsin, trypsin and papain digests of albumin, globulin, gelatin, casein, protamine, and tissue proteins from heart, liver and brain; also, an *Escherichia coli* digest of casein, and commercial products: Amigen (Mead), casein hydrolysate (Squibb) and pharmaceutical peptone (Cudahy). Staining reactions of the silver derivatives were tested on mammalian nervous tissue. The objective of finding a silver-protein compound that stained axis cylinders selectively was attained only with redissolved silver precipitates of pharmaceutical peptone and bacterially digested casein. It was concluded that the manner of degrading a protein prior to combining it with silver was the most important factor in determining the subsequent staining reaction.**

Soluble silver proteinates were first synthesized by Eichengrün (1898) to facilitate the use of silver as a therapeutic agent. He accomplished this by precipitating a solution of deuteroalbumose with silver nitrate, and dissolving the precipitate with a concentrated solution of protalbumose. Although the product obtained by this method was readily soluble in water, its silver ionization was of such low magnitude that it was not precipitated by the addition of chlorides. The lack of ionization, however, did not seem to impair the antiseptic properties of the silver. Silver proteinates (protargol, Bayer and Co.) were adopted for histological staining quite fortuitously by Regaud and Dubreuil (1903) while attempting to overcome some of the difficulties encountered in staining with silver nitrate. Not long afterwards they were introduced into neurological staining by Lugaro (1904) for the coloration of neurofibrillae. Further progress was not made until Bartelmez and Hoerr (1933) used a silver proteinate

[1]Publication No. 543.

method for sectioned tissue by substituting protargol for silver nitrate in Rogers' (1931) modification of the Bielschowsky technic. Bodian (1936) developed the on-the-slide reduced protargol method which is in general use today, and advocated the addition of metallic copper to the staining bath as a means of increasing the activity of the silver.

Silver proteinates have been supplied to the trade by several pharmaceutical houses, but only that supplied by the Winthrop Chemical Company under the trade name Protargol was satisfactory for neurological staining. The present work is a study of the factors which determine the staining properties of silver proteinates in the hope of clarifying relationships between these properties and methods of preparation.

Experimental Data

Procedure. The original procedure given by Eichengrün was used as a pattern for the synthesis of silver proteinates. A protein, or split protein product, was combined with a silver salt, and the resulting precipitate dissolved in a proteose solution. It was immediately evident that the protein material used for precipitation with the silver was the most important factor in determining the staining properties of the final product, whereas varying the proteose used for resolution of the precipitate had little effect. It was found also that saturating the protein solution with oxygen before precipitating it with silver increased the staining activity of the final solution. The general procedure adopted was as follows: Fifty milliliters of a 10% solution of protein material was saturated with oxygen by bubbling the gas through it. In some cases a 10% solution could not be obtained and so a saturated solution was used. The oxygenated protein material was then precipitated with an equal volume of 20% silver nitrate solution at room temperature. The supernatant was decanted and the residue washed several times in distilled water. The addition of 50 ml. of a 30% aqueous solution of pharmaceutical peptone (Cudahy)[2] redissolved the precipitate in most cases. This proteose was adopted for routine use, after trying several others, because of its constant composition from lot to lot and its ready availability. This preparation is a mixture of split protein products with a declared content of 51.7% proteose. The redissolved silver derivatives were then tested for their staining properties, although the solutions were much more concentrated than necessary. The dry silver proteinate could be obtained at this stage by precipitating with alcohol, washing with ether and drying in vacuo.

The tissues used for testing these stains were peripheral nerve, cerebellum and cerebral cortex of cat and monkey. This material was fixed in a modified Bouin's fluid (formalin, 10 ml.; glacial acetic acid, 5 ml.; picric acid, saturated aqueous solution, 50 ml.; ethyl

[2]Cudahy Packing Co., Omaha, Nebr.

alcohol, 40 ml.), embedded in paraffin, sectioned and mounted on slides in the usual manner. After staining for 48 hours the slides were rinsed for 30 seconds in distilled water and reduced in an aqueous solution of 5% sodium sulfite and 1% hydroquinone. Favorable preparations were compared to similar slides stained with protargol (lot No. N–051).

SOURCES OF PROTEIN AND STAINING REACTIONS

A wide variety of whole proteins and split protein products were employed for precipitation with silver. Variations both as to the elements stained and the quality of staining were seen when the different silver derivatives were tested for their staining properties.

1. *Whole Proteins.* Silver derivatives of the whole proteins used, namely, albumin, globulin, gelatin, casein, protamine and water soluble fractions from defatted mammalian heart, liver and brain were soluble only when the final proteose solution was alkalinized with 1 to 3 ml. of concentrated ammonia or sodium hyroxide. Presumably this resolution was due to the formation of complex ammoniacal or organic hydroxide silver ions, depending on the alkali used. Nevertheless, the minimum amount of alkali required was sufficient to inhibit the staining of axis cylinders, although myelin sheaths often stained intensely.

Recently, Moskowitz (1950) successfully used a mixture of silver nitrate and gelatin made soluble with sodium hydroxide for staining parasites. Such a stain was tried in our laboratories for nervous tissue but was unsatisfactory as the alkalinization was deleterious to the staining of axons.

2. *Enzymatically Digested Proteins.* Pepsin, trypsin and papin were used for enzymatically digesting the whole proteins mentioned above. One gram of commercial enzyme was used for 1000 g. of protein. Silver derivatives were readily soluble in concentrated proteose solutions, as were most silver precipitates of split protein. Digestion with pepsin gave a product whose silver derivative inconsistently stained all neural elements without specificity for any one component. The other two enzymes gave split proteins whose silver derivatives did not stain at all.

3. *Bacterially Digested Proteins.* It was accidentally found that the silver derivative of an unsterile solution of casein which had been allowed to stand for three weeks stained axis cylinders more specifically than any other silver proteinate. The organism responsible for the putrefactive changes was isolated and found to be a lactose-nonfermenting strain of *Escherichia coli.* Subsequent inoculations of sterile casein and other whole proteins with this pure culture gave similar results. However, the length of time required for bacterial digestion and the frequently encountered mutation of the bacterial strain made control of this procedure impractical.

4. *Commercially Split Proteins.* Three commercially prepared products were used also: amigen (Mead), casein hydrolysate (Squibb) and pharmaceutical peptone (Cudahy). Only the latter preparation formed a silver compound which consistently stained axons with any degree of selectivity. Silver derivatives of the other two products stained all neural elements.

Summary of Results

Variations in the staining reactions of silver proteinates are dependent upon the protein material employed for precipitation with silver. Generally it was found that the manner in which the protein was degraded was of more importance in determining the selectivity of the silver derivative than was the source of the protein. The only two split proteins whose silver derivatives consistently stained axons were pharmaceutical peptone and that obtained by bacterial digestion with *Escherichia coli.* Although preparations made with these products did not show absolute axis cylinder specificity they did at times show staining comparable to that seen with the original protargol. The silver content of the products made with these two split proteins was fairly constant, ranging from 9.0 to 11.0%. The pH of their solutions ranged from 3.0 to 4.5. The staining of neural elements at this degree of acidity was rather surprising because the pH of solutions of the original protargol is about 8.0. Some experiments with the staining reactions of silver derivatives of methylated and acetylated proteose have been made, but the behavior of such compounds needs further study before being reported formally.

In evaluating the present status of the general problem, the authors believe that it will be possible to synthesize silver-protein compounds whose selectivity can be predetermined to a greater degree than is possible at present. The complexity of the problem is against the finding of an easy solution.

This work was financed largely by a grant from the Biological Stain Commission.

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SILVER IMPREGNATION OF DEGENERATING AXON TERMINALS IN THE CENTRAL NERVOUS SYSTEM: (1) TECHNIC. (2) CHEMICAL NOTES

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Received for publication April 17, 1950

ABSTRACT.—**A new silver technic, tested on the brain of the rat, is described, especially suitable for demonstrating terminal degeneration within the central nervous system. It is a modification of the Glees method, designed to avoid use of tap water in preparing solutions. Some of the chemical principles underlying the process of reductive liberation of metallic silver from ammoniacal silver nitrate solutions are discussed.**

Since first applied by Hoff (1932), the method of studying central nervous connections in experimental material by silver impregnation of their degenerated synaptic end-portions has been employed on a steadily increasing scale. However, up to the present time a wide application of this research method has been seriously discouraged by technical difficulties.

Recently, Glees (1946) devised a modified Bielschowsky technic which has yielded valuable results in experimental studies on various brain regions (Glees, 1944; Glees, Meyer and Meyer, 1946; Clark and Meyer, 1948; Brodal, 1949). However, we were unsuccessful in an attempt to study hypothalamic connections in the rat by the Glees method, while the cerebral cortex was found to be somewhat resistant also. Since the Glees method involves the use of tap water in the preparation of the reducing fluid, we wish to present a modification which is independent of local variations in the chemical constitution of tap water. As shown by the illustrations, satisfactory pictures of terminal degeneration were obtained by this method in several telencephalic regions such as the neocortex and nucleus caudatus, as well as in the diencephalon. The method has not been tried on experimental material of other regions, but findings in normal sections of the lower brain stem and spinal cord indicate that it will succeed there on experimental material.

THE STAINING PROCEDURE

1. Fixation in 10% neutral formalin for 2 weeks up to 6 months.
2. Frozen sections of 15–20μ thickness are cut.

[1]Now at the research laboratories of J. R. Geigy AG, Basel, Switzerland.

3. Sections are demyelinated by being kept for 6 to 12 hours in 50% alcohol containing 1 ml. of strong ammonia (25% *ammonium hydricum*) per 100 ml. of alcohol. This treatment can be prolonged for some days without ill effect.
4. Sections are passed through at least three dishes of distilled water.
5. Sections are transferred for 12 to 24 hours into a solution composed of:

Silver nitrate	150 mg.
Distilled water	10 ml.
Pyridine	0.5 ml.

6. Without washing, sections are transferred for 2–5 minutes into an ammoniacal silver nitrate solution:

   450 mg. silver nitrate are dissolved in
   20 ml. of distilled water;
   10 ml. of pure ethyl alcohol, 97%, are added; the mixture is allowed to cool to room temperature. From calibrated pipettes
   2.0 ml. of strong ammonia (25% *ammonium hydricum*), and
   2.2 ml. of a 2.5% aqueous solution of sodium hydroxide are added.

   The solution is mixed thoroughly. The container is kept covered to prevent loss of excess ammonia.
7. Without further treatment, sections are transferred quickly into a reducing fluid:

10% alcohol	45 ml.
10% formalin[2]	2 ml.
1% citric acid[3]	1.5 ml.

   The sections expand on the surface and rapidly acquire a golden brown color.
8. Sections are transferred for 1 to 2 minutes into a 2.5% solution of sodium thiosulfate.

[2]Non-neutralized commercial formalin taken for 100%. The formalin used contained less than 0.03% formic acid.

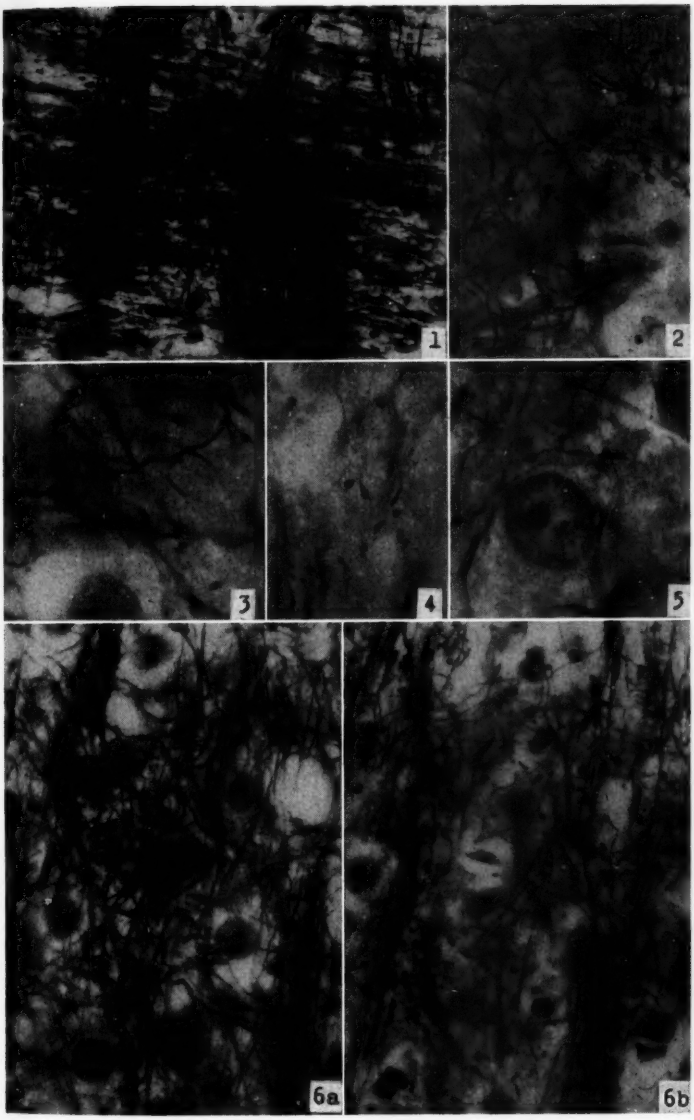
[3]1 g. of citric acid crystals per 100 ml. aqueous solution.

FIG. 1. — Antero-ventral nucleus of thalamus, showing massive degeneration of Vogt's septo-thalamic tract 3 days after lesion of supracommissural part of septal area. Notice contrast with normal capsula interna fibers. ×420.

FIG. 2. — Disintegrating fiber terminals in the inter-anteromedial cell mass of the thalamus. Degeneration time 3 days. ×720.

FIGS. 3–5. — Terminal degeneration in medial region of hypothalamus (3), periventricular region of hypothalamus (4) and nucleus caudatus (5). Degeneration time 3 days. ×1200.

FIG. 6. — Normal aspect (6a) of fourth layer of frontal cortex, and (6b) same layer as appearing 4 days after left-sided lesion in rostral part of internal capsule. 6a and 6b represent identical points of right and left side respectively as seen in one and the same section. ×600.


1
2
3
4
5
6a
6b

They are washed in at least three dishes of distilled water. Contamination of this wash water by traces of the ammoniacal silver nitrate solution should be avoided, as it is liable to cause silver precipitates on the sections.

9. Finally, sections are passed rapidly through the graded alcohols and xylene. Neutral synthetic media such as clarite or caedax are preferred for mounting.

Throughout the procedure, a glass rod should be used for transferring the sections.

Discussion

Fixation and Embedding. In full agreement with Glees (1946), neutral formalin was found superior to other fixatives. Formalin fixation for more than 6 months, however, diminishes the effectiveness of the staining procedure, and brain tissue fixed for more than a year is usually refractory. Concerning embedding media, by far the best results were obtained in frozen sections. Celloidin embedding is less desirable, and paraffin material is unsuitable.

Since a more detailed knowledge of the physico-chemical aspects of silver deposition in organic material may lead to further improvement of neurological silver technics, some chemical data concerning the solutions employed in the process of impregnation will follow. An account of other important aspects of silver impregnations will be found in the paper of M. Seki (1940).

1. *Ammoniacal silver solutions.* As already found by Prescott (1880), Reychler (1883, 1895) and Draper (1886), addition of ammonia to sufficiently concentrated solutions of silver nitrate leads to precipitation of silver oxide which is progressively and completely redissolved by further addition of ammonia until the ratio is reached of approximately 2 molecules of ammonia to 1 molecule of silver nitrate. Bodlaender and Fittig (1901), continuing upon previous studies of Reychler (1895), Konowaloff (1898) and Berthelot and Delépine (1899), demonstrated the presence of silver in such solutions in the form of a complex cation,

$$[Ag(NH_3)_2]^+ \qquad (I)$$

Britton and Wilson (1933) demonstrated the formation from NH_3 and $AgNO_3$ of the complex salt silver-ammonia nitrate

$$[Ag(NH_3)_2]NO_3 \qquad (II)$$

They showed that this salt is not appreciably decomposed by addition of more ammonia, and thus may be regarded as the salt of a base

$$[Ag(NH_3)_2OH] \qquad (III)$$

which is stronger than ammonia. The existence of this base was actually demonstrated by electrometric titration of a solution of silver oxide in ammonia. It is important that the strength of the base (III) was found to be comparable with that of sodium hydroxide.

2. *Instability of the complex* $[Ag(NH_3)_2]^+$. The complex (I) formed in the ammoniacal silver nitrate solution is in equilibrium with its components according to the equations

$$k_1 = \frac{[Ag(NH_3)^+]\ [NH_3]}{[Ag(NH_3)_2]^+} \qquad \text{(IV)}$$

and

$$k_2 = \frac{[Ag^+]\ [NH_3]}{[Ag(NH_3)^+]} \qquad \text{(V)}$$

Since the existence of a mono-ammonia-metal complex ("monammine complex ion") as indicated in equation IV was postulated only recently (Bjerrum, 1941; Vosburgh and Stockdale Mc Clure, 1943), only the instability constant or complexity constant K (now called "overall complexity constant") is mentioned in the older literature:

$$K = k_1 \cdot k_2 = \frac{[Ag^+]\ [NH_3]^2}{[Ag(NH_3)_2{}^+]} \qquad \text{(VI)}$$

According to Vosburgh and Stockdale Mc Clure (1943), $k_1 = 4.3 \cdot 10^{-4}$ and $K = 6.2 \cdot 10^{-8}$, while (Bjerrum, 1941) the instability constant k_2 of the monammine-ion equals $4.8 \cdot 10^{-4}$.

It is evident from these figures that the concentration of non-combined, "free" silver (Ag^+ in equation VI) in ammoniacal silver solutions is extremely low. This is a significant fact, since this concentration determines the electro-chemical potential of the solution, and, in accordance with the equilibrium constants, also the decomposition of the silver complex during reduction. It is important in this connection that addition of ammonia to silver nitrate solutions beyond the point where an initially formed precipitate of silver oxide has been dissolved completely, with formation of the complex salt (II), lowers the concentration of silver ions ("free silver") (Kohlschütter, 1912; Britton and Wilson, 1933). This decrease can be explained thus: the excess of the weak base ammonia does not decompose the salt $[Ag(NH_3)_2]NO_3$ into its free base and ammonium-nitrate; instead, in accordance with the law of mass action, it increases the stability of the complex $[Ag(NH_3)_2]^+$, thereby lowering the concentration of free silver. As shown by Kohlschütter, continued addition of ammonia may even suppress the concentration of free silver to levels where reductive liberation of metallic silver by formaldehyde is no longer possible.

An entirely different effect on ammoniacal silver nitrate solutions is shown by strong bases, such as sodium hydroxide. They tend to liberate the base (III) from the complex salt (II). Base (III) in turn, by releasing the decomposable complex ion $[Ag(NH_3)_2]^+$, increases the concentration of free silver in the solution. Accordingly, NaOH is able to negate the effect on free silver concentration of excess ammonia. (Kohlschütter, 1912).

As the antagonistic effects of excess ammonia and sodium hydrox-

ide respectively were expected to furnish a valuable means of controlling the concentration of free silver in ammoniacal silver solutions, experiments were performed to determine the ratio of silver nitrate, ammonia and sodium hydroxide optimum for the demonstration of terminal degeneration. For the brain of the rat, the formula given above yielded the best results.

Changes in the formula were observed to affect profoundly the subsequent stain. If for instance, appreciably less sodium hydroxide is added, e.g. 1.5 ml. instead of 2.2 ml. as indicated, staining is too weak and many fibers are impregnated irregularly. Too high concentrations of sodium hydroxide, e.g. 4 ml., will suppress the impregnation of degenerating fibers and cause overstaining of the "background", although normal fine fibers may be shown in great detail. Addition of still more alkali may prevent impregnation of degenerating fibers completely, while massive and apparently unselective silver depositions occur.

Reducing Fluid

The use of a very dilute formalin solution in 10% alcohol proved to be preferable to the conventional much stronger aqueous formalin. Acidification of the solution is essential in avoiding unselective silver deposition. For this purpose citric acid was found to be the most suitable of the various acids tried. Excessive acidification suppresses the reduction process, but in suitable concentration citric acid markedly improves the selectivity of the impregnation, presumably by buffering the liberation of metallic silver from the ammoniacal silver nitrate solution.

Comments

Although the above described method yielded remarkably constant results in the rat's brain, modifications of the technic may prove necessary for work on other animals. Such modifications will probably be achieved most readily by changing suitably the ratio of ammonia and sodium hydroxide in the ammoniacal silver solution.

N.B. Silver ammonia hydroxide has an erratic tendency to decompose, yielding the violently explosive silver amide:

$$2[Ag(NH_3)_2]OH \rightarrow Ag_2NH + 3NH_3 + 2H_2O.$$

The ammoniacal silver solution must therefore be prepared immediately before use, and remnants should be discarded.

We wish to thank Mr. and Mrs. William G. Lucas for valuable technical assistance. We are also indebted to Mr. Gilbert Michel for helpful criticism in the preparation of the manuscript.

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STAINING NEUROGLIA WITH SILVER DIAMMINO-HYDROXIDE AFTER SENSITIZING WITH SODIUM SULFITE AND EMBEDDING IN PARAFFIN[1]

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Received for publication May 25, 1950

ABSTRACT.—**Pieces of brain are fixed in formalin ammonium bromide for about 4 days at room temperature, and given the following treatment: After washing, dehydrating and clearing, embed in paraffin, section and mount. Deparaffinize sections and pass through graded alcohols to water. Sensitize in 5% sodium sulfite for 2 hours, wash in distilled water and impregnate with silver diamminohydroxide solution 2–5 minutes at room temperature. Reduce in 2% formalin, wash in distilled water and tone in gold chloride. Fix in 5% hypo, counterstain with 1% picric acid, dehydrate and cover in balsam. Equally good results are obtained by impregnating with Hortega's strong silver carbonate. The microglia, oligodendroglia, fibrous and protoplasmic astrocytes in the cat, rabbit, newborn and adult human are successfully stained by this method.**

According to Penfield (1932) the gold sublimate method, developed by Cajal 1913, gave neurohistologists the first highly selective neuroglial stain. Del Rio Hortega (1942, 1943) made a general revision of the methods for staining neuroglia, especially those in which silver carbonate is used. The gold sublimate and various silver impregnation methods for neuroglia, developed chiefly by the Spanish investigators, are all made on frozen sections. In spite of its common usage, there are serious objections to this method of sectioning. Some of these objections were discussed by Shanklin (1943).

For some years we have been studying, in this laboratory, methods for silver impregnation following paraffin embedding and sectioning. A report was published by Shanklin (1943) on the use of Hortega silver carbonate for staining pituicytes after paraffin embedding. More recently we (Nassar and Shanklin, 1950) have succeeded in staining the parenchymatous cells of the pineal by a modified Hortega method after paraffin embedding.

Weil and Davenport (1933) used an ammoniated silver solution for staining oligodendroglia and microglia in celloidin sections. Ammoniated silver was used also by Davenport, Windle and Beech

[1]This investigation was supported by a grant from the Committee for Research in Endocrinology, National Research Council.

(1934) for staining nerve cells and by Lillie (1946, 1948) for staining reticulum and collagen.

Our method is as follows:

1. Fix small fresh pieces of the central nervous system in formalin ammonium bromide for 4 days at room temperature (Del Rio Hortega, 1942, p. 178).

Formalin (Merck, blue label 40%)	70 ml.
Ammonium bromide	14 g.
Distilled water	680 ml.

2. Wash 10 hours in distilled water to which 30 drops of strong ammonia water are added for each 100 ml. of water, and cover the jar.

3. Wash in two changes of distilled water for 1–2 hours in each; dehydrate with alcohol.

4. Clear in cedar oil followed by xylene for half an hour.

5. Infiltrate with paraffin for 3–4 hours, embed, section at 10–15μ and fix to slide by the albumin water method.

6. Remove the paraffin with xylene, dehydrate with alcohol and pass through three changes of distilled water 1–2 minutes each.

7. Sensitize by placing the slides in 5% sodium sulfite for two hours.

8. Pass quickly through three changes of distilled water.

9. To prepare silver diamminohydroxide solution place 1 ml. 28% ammonia water in a small flask and add 7 or 8 ml. 10% silver nitrate rapidly. Continue to add 10% silver nitrate drop by drop shaking between each addition to clear the solution until a faint permanent turbidity remains after the last drop added. This takes a total of 9 to 10 ml. silver solution. Then dilute the resultant solution with an equal volume of distilled water (Lillie, 1946, p. 70).

10. Impregnate by immersion in the silver solution (2) at room temperature for 2 to 5 minutes; the time is varied until the optimum

[2]Equally satisfactory results were obtained by impregnating with strong silver carbonate after Del Rio Hortega 1942, p. 179. The photomicrographs are all from silver carbonate impregnated sections.

PLATE 1

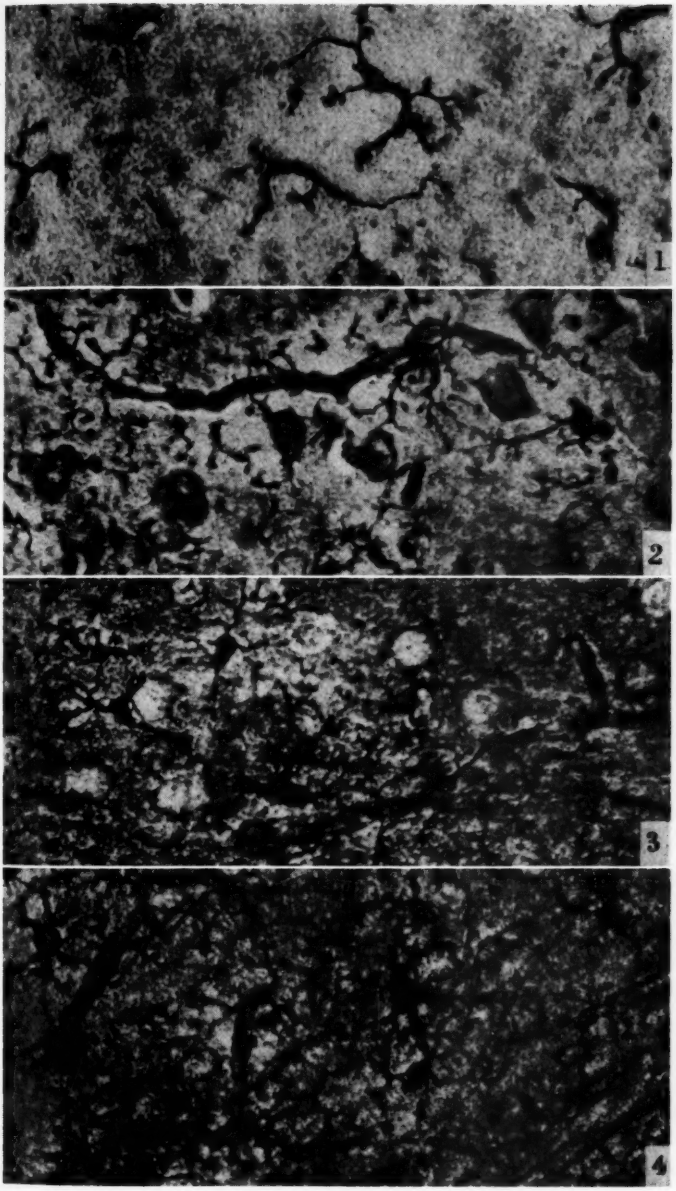
All figures are untouched photomicrographs. × 600.

FIG. 1 — Molecular layer of rabbit cerebellum showing microglia.

FIG. 2. — Cerebral cortex of rabbit with microglia.

FIG. 3. — Cerebral cortex of rabbit. The more centrally placed cell with profuse branching is a protoplasmic astrocyte; smaller ones with dark nuclei are microglia.

FIG. 4. — Cerebral cortex of rabbit. The cell in the upper left corner with process attached to a blood vessel is a fibrous astrocyte. The other two large cells with profuse branching are protoplasmic astrocytes. The smaller darker cells are microglia.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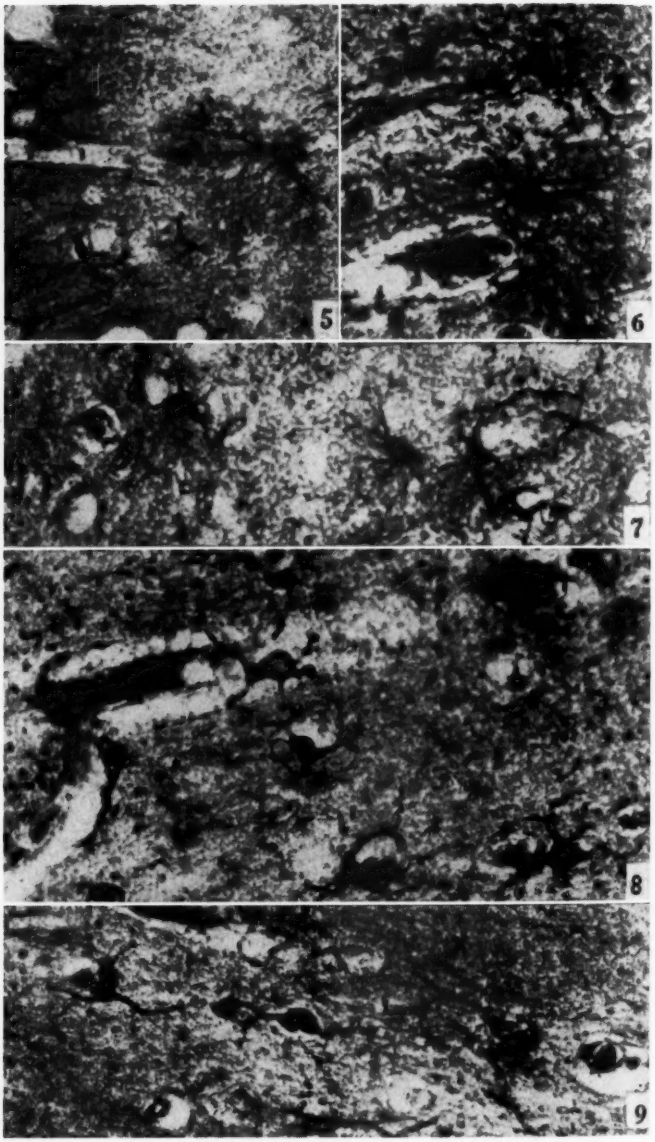
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is determined. The silver solution will keep for several days but should be changed frequently after use.

11. Dip in distilled water 1 or 2 seconds.

12. Reduce for one minute in 2% neutral formalin (Merck, blue label) agitating gently. The formalin should be changed frequently.

13. Wash in distilled water for one minute.

14. Tone in yellow gold chloride (1 g. to 500 ml. distilled water) a few seconds to one minute. This step must not be prolonged beyond the exact time needed.

15. Fix in 5% hypo for 1–2 minutes.

16. Wash in tapwater and counterstain lightly with 1% picric acid.

17. Dehydrate in alcohol, clear in xylene, mount in neutral balsam and cover with cover slips.

When pieces of the central nervous system were impregnated with silver diamminohydroxide or with strong Hortega silver carbonate after sensitizing with sodium sulfite, the astrocytes, microglia and oligodendroglia were well stained. When the same silver methods were used without previous sensitizing with sodium sulfite the results were unsatisfactory.

Summary

Fibrous and protoplasmic astrocytes, microglia and oligodendroglia have been successfully stained after fixation in formalin ammonium bromide, sensitizing in sodium sulfite and impregnating either with silver diamminohydroxide, or with strong Hortega silver carbonate, following paraffin embedding, in the rabbit, cat, newborn and adult human central nervous system.

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PLATE 2

Fig. 5. — Cerebral cortex of cat demonstrating a protoplasmic astrocyte.

Fig. 6. — Cerebral cortex of adult human showing a protoplasmic astrocyte.

Fig. 7. — Cerebral cortex of newborn human with fibrous astrocytes.

Fig. 8. — Cerebral cortex of adult human including fibrous astrocytes.

Fig. 9. — Cerebral cortex of adult human. The x is among the processes of a protoplasmic astrocyte. The smaller darker cells are microglia.

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EFFECT OF FORMALIN FIXATION ON THE ACTIVITY OF FIVE ENZYMES OF RAT LIVER[1]

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Received for publication June 21, 1950

ABSTRACT.—**The effect of formalin fixation on the enzymatic activity of five enzymes in rat liver was studied as a function of time and temperature. β-glucuronidase was found to be most resistant to the inactivating effects of formalin. At a time when fixation was satisfactory for preparing frozen sections (24 hours), considerable enzymatic activity was present in decreasing order; β-glucuronidase, sulfatase, acid phosphatase, esterase, and alkaline phosphatase.**

INTRODUCTION

In order to demonstrate intracellular enzymatic activity histochemically, the type of fixation which enzymes will tolerate becomes very important. This is particularly the case with certain tissues which do not yield good sections by a fresh frozen technic, and in the case of most sections of fresh tissues where incubation with the substrate must be conducted at 37°C. for many hours. When fixation can be performed in organic solvents without too extensive inactivation of enzymatic activity, paraffin-embedded sections can be prepared. This has been possible with alkaline phosphatase (Gomori, 1939; Manheimer and Seligman, 1948), acid phosphatase (Gomori, 1941; Seligman and Manheimer, 1949), and esterase (Nachlas and Seligman, 1949a). Acetone-fixation for 24 hours and embedding in paraffin reduced the enzymatic activity of alkaline phosphatase 80% (Stafford and Atkinson, 1948), of acid phosphatase 95% (Stafford and Atkinson, 1948), and of esterase 60% (Nachlas and Seligman, 1949a). Alcohol was even more destructive of acid phosphatase (Seligman and Manheimer, 1949) and esterase activity (Nachlas and Seligman, 1949a). In developing histochemical methods for sulfatase and β-glucuronidase (Seligman, *et al*, 1949) it was found that fixation in organic solvents and embedding in paraffin was so destructive of enzymatic activity that satisfactory methods based on this technic could not be evolved. However, when the effect of formalin-fixation

[1]This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, (in part) by a grant from the Massachusetts Division of the American Cancer Society, and (in part) by an institutional grant to Harvard University from the American Cancer Society.

[2]Research fellow of the National Cancer Institute.

was explored, it was found that both these enzymes tolerated brief fixation in 10% formalin very well. The effects of formalin-fixation on the activity of these five enzymes in the liver of the rat are presented below.

Method

Pieces of liver from adult Wistar rats were cut 2 mm. thick, weighed within 15–30 minutes after death of the animal, and placed in 50–100 ml. formalin (10%) buffered (Lillie, 1948) at pH 7.0 with 4.02 g. monosodium phosphate and 16.37 g. disodium phosphate. After fixation, each piece was washed for 5 minutes in running tap water, and was then homogenized in a motor-driven glass homogenizer for 3 minutes. Various dilutions of the homogenate were prepared so that the final concentration of liver in milligrams per milliliter corresponded to that shown in Table 1 for each enzyme studied. This was necessary because of the great difference in activity of each enzyme in order to bring within the range of the colorimeter the quantity of dye which was produced from the substrates after incubation for the periods shown in Table 1. The enzymatic activity of aliquots of unfixed liver was also determined after similar dilution. This activity was taken to be 100% in each case.

Table 1. Protocol Data

Enzyme	Concentration of liver in homogenate	Substrate	pH	Buffer	Period of incubation at 37.5°C.
	mg./ml.				hours
Alkaline phosphatase	10.0	Monosodium β-naphthyl* phosphate	9.1	0.1 *M* Veronal	2.0
Acid phosphatase	1.0	Monosodium β-naphthyl* phosphate	5.0	0.2 *M* Acetate	1.5
Esterase	0.1	β-Naphthylacetate	7.4	0.1 *M* Veronal	0.5
Sulfatase	10.0	Potassium 6-bromo-2-† naphthyl sulfate	5.0	0.2 *M* Acetate	24.0
β-Glucuronidase	10.0	Brucine salt of 6-bromo-† 2-naphthyl glucuronide	4.5	0.2 *M* Acetate	24.0

*Method of preparation is given by Friedman and Seligman (1950).
†Methods of preparation to be published by Seligman.

Each substrate (10 mg.) was dissolved in 50 ml. of water and 50 ml. of the appropriate buffer (Table 1). Naphthyl acetate was first dissolved in 2 ml. acetone and added to 48 ml. of water and 50 ml. of buffer as previously described (Nachlas and Seligman, 1949b).

The substrate solution (5 ml.) and the diluted liver homogenate (1 ml.) were placed in a 20 ml. test tube and incubated at 37.5°C. for the period given in Table 1. The pH was raised to 8.5–9.2 by addition of 4–5 drops of 10% sodium carbonate solution. A freshly prepared solution (1 ml.) of tetrazotized diorthoanisidine[3] (4 mg/ml.) was added. A few minutes later, 1 ml. of 40% trichloroacetic acid was added to facilitate extraction of the dye from protein and to stop the decomposition of the excess diazonium salt. Ethyl acetate (10 ml.) was added and the tube was thoroughly shaken to extract the azo dye. The emulsion which formed was broken by centrifugation at 2000 rpm. for 15 minutes. The ethyl acetate (5 ml.) was transferred with a pipette for measurement of its color density (Nachlas and Seligman, 1949b; Seligman, Nachlas and Mollomo, 1949; Seligman and Nachlas, 1950) with a photoelectric colorimeter (Klett) through a 540 mμ filter. The percent of enzymatic activity remaining in the

TABLE 2. PERCENT OF ENZYMATIC ACTIVITY REMAINING IN RAT LIVER AFTER FIXATION IN 10% FORMALIN.

(buffered at pH 7.0)

Time and temperature of exposure	Number of experiments	Alkaline Phosphatase	Acid Phosphatase	Esterase	Sulfatase	β-Glucuronidase
2 hr. 4°C.	5	73%	79%	82%	74%	86%
24 hr. 4°C.	7	26	55	35	58	87
48 hr. 4°C.	3	30	21	50	12	83
1 hr. 25°C.	3	48	58	67	59	100
2 hr. 25°C.	3	35	40	53	61	89
1 hr. 37°C.	3	24	41	27	63	105
2 hr. 37°C.	3	13	27	23	41	91

tissue after fixation was obtained from the ratio of the color density per unit weight of tissue of the tube containing fixed liver to that containing unfixed liver.

RESULTS

In Table 2, is shown the effect of fixation of rat liver in neutral formalin (10%) at 4°, 25°, and 37°C. for various periods of time, on the percent of enzymatic activity which remains in the tissue. The results with each of five enzymes are the average of 3–7 experiments. Incubation of liver for 1 and 2 hours at 55°C., or in 40% formalin

[3]Commercially available in stable powder form as naphthanil diazo blue B, Dupont de Nemours & Co., Boston. The powder contains 20% tetrazotized diorthoanisidine. This reagent, β-naphthyl phospate, and naphthyl acetate may be purchased from Dajac Laboratories, Monomer-Polymer, Inc., 3430 W. Henderson Street, Chicago 18, Illinois.

produced nearly complete inactivation of enzymatic activity and these results are not included in the table. Other experiments not included in Table 2 consisted in washing the tissue blocks after fixation for periods up to 24 hours. The results were the same. Variation in the thickness of the tissue blocks gave similar results when fixation for 24 to 48 hours was employed. With periods of fixation as short as 1 and 2 hours, incomplete fixation of the center of thick blocks resulted in higher figures.

In general the optimum conditions for fixation of tissue for histochemical procedures appeared to be 24 hours at 4°C., in that considerable enzymatic activity remained, and the tissues were sufficiently hard for making good frozen sections. The exceptions to this were β-glucuronidase, which was highly resistant to inactivation by formalin under all conditions (Table 2) and esterase, which is very abundant in tissue (Nachlas and Seligman, 1949c) and therefore can afford a greater degree of inactivation without interfering with the efficacy of the histochemical method (Nachlas and Seligman, 1949c).

The percent of enzymatic activity remaining after formalin fixation for 24 hours at 4°C. in the case of alkaline phosphatase and esterase was similar to that remaining after fixation in organic solvents and embedding in paraffin (Stafford and Atkinson, 1948; Nachlas and Seligman, 1949a), and in the case of acid phosphatase was very much higher (55%) as compared to that after acetone fixation (20%) and embedding (6%) in paraffin (Stafford and Atkinson, 1948). However, these figures give no indication of the degree of inactivation of other varieties of acid phosphatase such as that in prostatic epithelium (Seligman and Manheimer, 1949). Recent experiments of Seligman and Cohen, show that the alkaline phosphatase which survives fixation in organic solvents is different in distribution from the alkaline phosphatase which survives fixation in formalin.

In other experiments designed to test the activating influence of metallic ions after fixation in formalin for 24 hours at 4°C., a block of tissue was soaked in solutions containing magnesium chloride, calcium chloride, and ferric ammonium sulfate (1 mg./ml.) for 1 hour.

Alkaline phosphatase was activated about 35% by magnesium chloride, acid phosphatase was activated about 50% by magnesium chloride and a few per cent by calcium chloride, and sulfatase was activated 50% by magnesium chloride and 25% by calcium chloride and ferric ammonium sulfate. Activation of alkaline phosphatase in aged specimens with magnesium ions has been reported (Fischer and Greep, 1948).

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STAINING OF RADIOAUTOGRAPHS WITH METANIL YELLOW AND IRON HEMATOXYLIN[1]

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Received for publication May 3, 1950

INTRODUCTION

The purpose of this paper is to present a staining procedure for radioautographs prepared by mounting tissue sections directly on photographic emulsions. Heavy and uneven absorption of stains by

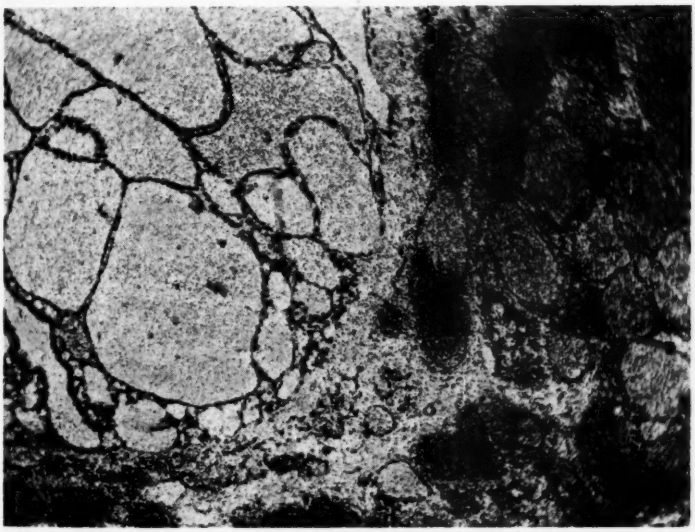

FIG. 1. — Radioautograph. Macrofollicles of the thyroid gland (human) showing slight to no concentration of radioiodine[131]. Adjacent normal follicles show slight to heavy concentration of the isotope. Kodak Medium Lantern Slide emulsion. Metanil yellow and iron hematoxylin stain. ×90.

the emulsion gelatin precludes the use of routine hematoxylin and eosin methods. Metanil yellow and iron hematoxylin have been employed and found to give uniformly satisfactory results.

[1]Jointly supported by the Office of Naval Research Contract No. N6-ori-99, Task Order No. 1 and the Atomic Energy Commission.

[2]Special Research Fellow (1948–1949) — National Cancer Institute.

[3]Special Fellow in Pathology — American Cancer Society.

Materials and Method

Surgical and autopsy specimens from patients having recently received radioiodine (I^{131}) and tissues from laboratory animals to which radioiodine, radiosulfur (S^{35}) or radiophosphorus (P^{32}) had been administered were used in this study. The tissues were fixed in formaldehyde solution or Bouin's fluid. Zenker's or Helly's fluids were found to be unsuitable as they contain mercuric chloride which causes reduction of the photographic emulsion. After adequate fixation the tissues were processed through alcohol, dioxane and paraffin. Sections were cut at 7–10 μ. The ribbons were then transferred to the darkroom, placed in a waterbath and floated onto a photographic plate (Evans, 1947; Endicott and Yagoda, 1947). X-ray film, Kodak Medium Lantern slides and Kodak Nuclear Track plates of varying thickness have been used for this purpose. Upon drying the sections remain permanently attached to the photographic emulsion. For each isotope the processing fluids were examined by Geiger counter to insure that there was no significant loss of the radioactive material from the tissue.

After proper photographic exposure, deparaffinization, development, fixation, washing and drying of the radioautograph it is stained as follows:

1. Metanil yellow. 0.25% aqueous solution, 5–15 seconds. This solution may be used many times without losing its staining properties and apparently keeps indefinitely.
2. Wash in tap water until the slide is pale yellow – about 15 seconds.
3. Iron hematoxylin (modified from Weigert, see Mallory, 1938); 1–3 minutes. Solution A. Hematoxylin: 1 gram of the powdered form dissolved in 100 ml. of 95% alcohol. Solution B. Ferric chloride (50% aqueous solution), 5 ml.; distilled water, 95 ml.

   Equal parts of solutions A and B are mixed and allowed to stand for 2 to 3 weeks before use. During this time the color of the stain changes from the purple of the fresh mixture to dark brown. The fresh solution is too strong for immediate use since it darkens the emulsion gelatin. The stain should be filtered before use. Sixty milliliters (one Coplin jar) is adequate for about 20 radioautographs.
4. 70% alcohol; 3 changes, 5 seconds each.
5. 95% alcohol; wash, 5 seconds.
6. Acetone; wash, 5 seconds.
7. Acetone and xylene, equal parts; wash, 5 seconds.
8. Xylene; 3 changes.
9. Mount in synthetic resin.

Comments

The emulsion is stained a uniform light yellow from which reduced black silver granules stand out well (Fig. 1). Heavy staining of the emulsion gelatin by hematoxylin is inhibited by first using metanil yellow. Emulsions containing large amounts of gelatin take up more stain and often show uneven distribution of it throughout the plate. Emulsions of lower gelatin content, such as the Nuclear Track plates are, therefore, more satisfactory for this method.

Colloid, cytoplasm and connective tissue elements are stained by metanil yellow in various shades from light yellow to light brown.

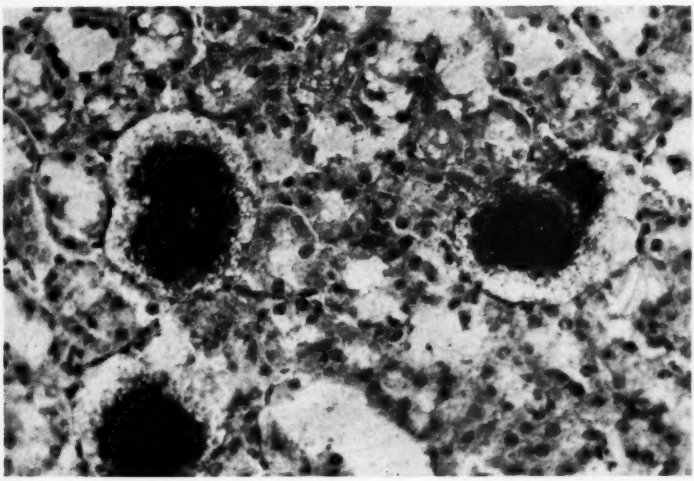

Fig. 2. — Radioautograph. Mouse kidney showing localization of radiosulfur^{35} in the glomeruli. Animal had been given anti-kidney serum labelled with the isotope. Kodak Nuclear Track Plate (NTB) emulsion. Metanil yellow and iron hematoxylin. ×120.

Cell nuclei are stained deep blue to black by the iron hematoxylin (Fig. 2). The stain has been used in an extensive study of radioautographs of thyroid carcinoma (Fitzgerald, 1948; Fitzgerald and Foote, 1949; Fitzgerald, Foote and Hill, 1950) and has been found satisfactory for radioautographs of tissues from all organs in man and several laboratory animals to whom radioisotopes had been administered (Pressman, Eisen, and Fitzgerald, 1950).

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WRIGHT'S STAIN FOR CHLOROPLASTS AND TISSUE SPREADS

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Received for publication Feb. 9, 1950

ABSTRACT.—**Wright's stain, including the entire Wright method as used in hematology, is applicable to fresh animal and plant tissues which may be spread with rapid drying. Particularly are the chloroplasts and vacuole plastids of *Nitella* well prepared by: pricking open a blotted, uninjured (streaming) cell upon a dry slide, thus bursting out both chloroplasts and other plastids which spread themselves; further teasing of more plastids from the cell membrane upon an adjacent dry region of the slide. Also Tunicate zooids blotted dry in transference to a slide may be torn open and the various tissues teased and lifted about on the slide with excellent spreading of cells. Rapid drying with gentle heat, well timed fixing and staining and differentiation all contribute to results which not only show normal cellular differentiations but which are very valuable in showing experimental cytological changes.**

One of the most sensitive stains known, Wright's polychromatic, is being largely neglected except for its use in hematology. To be sure, Lillie (1944, 1948) has used Wright's stain and other thiazin eosinates for staining sections of fixed tissue; but the method of applying the stain to sections is entirely different from the one used in hematology and hence gives quite different results. As a matter of fact, it is not only the stain itself that is valuable for other material besides blood, but the entire method by which it is applied in hemotology is well adapted to certain other materials. This method involves: rapid drying of tissue; simultaneous fixation with methanol and application of the compound stain; plus a radically induced differentiation technic.

What the stain accomplishes in hematology is important: it indicates normal and pathological states and changes in cytoplasmic basophilia, acidophilia, or polychromatophilia; it shows nuclear sculpturing details by color differentiation in non-dividing cells; it is sensitive for cellular granules. We know enough about its range, from its behavior in hematological variations, to be able to use it profitably wherever possible, especially in experimental work where general cytoplasmic and more specific cell inclusion changes may occur.

Wright's method is generally applicable for cytological work wher-

ever a material may be spread (not smeared) and dried rapidly. Where cells separate well and where there is neither an excess of water in tissue fluid nor a concentrated protein serum, conditions are favorable for a good spread of uninjured cells and an excellent stain. It is decidedly worthwhile to examine many types of fresh tissues to determine whether or not they are suitable for Wright preparations.

Especially in botany no text-book on technic mentions Wright's stain. The author finds that it produces excellent preparations of chloroplasts, which are highly desirable now when chloroplasts are being studied under many experimental conditions where a sensitive indicator technic is necessary.

Three species of *Nitella* have thus been studied in experiments involving a carcinogenic agent, and all material has been prepared using Wright's technic. Control and experimentally changed chloroplasts differ markedly in what appear to be valuable and delicately stained preparations, comparing well for size and shape with chloroplasts studied *in situ* in living, uninjured cells.

Method

A *Nitella* cell, carefully isolated from the plant, is effectively blotted in transference to the slide on a slip of filter paper. Then upon the dry slide the uninjured streaming cell is pricked. It bursts out its sap and chloroplasts in effective spread. The deflated cell membrane is then lifted with needles to an adjacent dry region and torn, releasing more chloroplasts with a small amount of sap. Areas of the cell wall are arranged smoothly; so that no portion of the cell nor its contents are lost in the preparation.

By thus spreading the cell fluid about, there is no excess in any region and the preparation may be dried rapidly with gentle heat; any very slight drop of sap drains off the critical region without disturbing either the spread of the chloroplasts or the interesting vacuole plastids which have never before been so favorably prepared. The cell wall stains admirably, and even external symbiotic algae are well preserved and stained.

The technic of staining and differentiation is precisely as for a blood film. In fact, a perfect routine hematological preparation of normal blood should be produced as control for both stain and timing in the methods, serving thus as technic criterion of the best the method may be expected to produce in new materials.

Many genera and species of Tunicate material produce handsome preparations if each living zooid is blotted before it is torn open and spread. Numerous types of uninjured cells readily become isolated as the tissues are lifted about and gently teased in the distributed body fluid. Drying must be accomplished as rapidly as possible, without over-heating. Fixation appears to be perfect, with no distortion, and

staining is delicate with highly diverse reactions in different tissue cells.

Results

The chloroplasts of normal *Nitella* are basophilic and of marked alveolar structure. It is possible that much material is soluble in the methyl alcohol of the stain and that the delicate structures thereby revealed represent a framework with certain important granules which may be the grana of recent electron microscopic investigation of chloroplasts by Granick and Porter (1947). What Granick (1949) calls the coherent structure of the chloroplast as independent of other cytoplasmic structure is admirably demonstrated.

Large, quite homogeneous appearing vacuole plastids which are noted in the streaming of the living cell vacuole are brilliantly eosinophilic, as a whole. However, they prove to be complex with a central raphe, usually colorless; arrangements of both basophilic and eosinophilic granules; and bilaterally distributed lacunae varying, according to experimental conditions, from colorless to a brilliant robin's egg blue.

The staining of Tunicate material is so critical that species differences are readily noted in comparative studies of the same cells.

No great success has yet been obtained with the method for Arthropod, Crustacean and Molluscan forms. The body fluids do not permit of either the type of spread necessary nor of rapid drying; and thus there is little clean-cut staining. Often even the most isolated blood elements are not perfectly demonstrated although brilliantly colored. The thick body fluid takes some of the stain.

Teased Coelenterate material gives strikingly polychromatic and brilliant cellular staining; but again a mucus consistency of the body fluid interferes so that few cells prove to be valuable for study.

Annelid material produces excellent results only for blood elements, for which Dawson (1933) has used Wright's stain and technic.

It must not be supposed that, either in spreading or in staining, Wright preparations are short-cuts over any type of mass tissue preservation and staining. The spreading, drying and staining of adequate amounts of material are time-consuming; each slide must be individually treated in every respect. Simply testing for suitable material is rapid, however.

Conclusions

Wright's stain and technic is valuable for showing cytoplasmic changes in various tissues and cell inclusions. Just as in hematology anemias of various types are demonstrated by chrome reactions to which Wright's stain is sensitive, so, in other tissues, does Wright's stain reveal cytoplasmic differentiation and experimental change.

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PREPARING DRY MOUNTS OF MOSQUITO LARVAE AND LARVAL SKINS WITHOUT USING ABSOLUTE ALCOHOL

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Received for publication April 25, 1950

Permanent preparations of mosquito larvae and larval skins can be made either as "dry" mounts or as "wet" mounts. The dry method involves complete dehydration through graded alcohols and final preservation in some water-free mounting medium, like canada balsam or euparal, which eventually dries up. The wet method on the other hand obviates the usually time-consuming process of dehydration, because the object is transferred directly from water to a watery mounting medium, like chloral gum (Puri, 1931) or lactophenol (Gibbons, 1930), the preparation being subsequently sealed with some water-proof substance such as varnish or zinc white cement. One serious disadvantage which detracts from this method, however, is that, unless frequently attended to, the preparation may be spoiled in the long run as a result of the entry of air into the mount through fissures formed in the protective ring, or of the cement itself oozing into the medium. Dry mounts are far more durable and would gain preference provided the tedium of dehydration through graded alcohols could be avoided without damage to the specimen. Wanamaker (1944) and Bhatia (1949) have each described such an improved method for mosquito larvae, which however requires the passage of the specimen through absolute alcohol or 95% alcohol for a short time prior to mounting. The dearth of absolute alcohol has compelled the present author to seek a simple, but efficient method in which the use of absolute alcohol could be eliminated. The technics described below fulfill these objectives and have given satisfactory results:

FOR LARVAE

1. Kill larvae by dropping them in water brought to boil.
2. Place the dead larvae in a watch glass or petri dish under a binocular microscope and, by means of sharp needles, nick each at two or three places laterally at the junction of the segments.
3. With a curved needle transfer the larvae, one by one, into a specimen tube containing a fixing-clearing fluid[1] of the following

[1]This fluid should not be left open, as it has a tendency to absorb moisture and turn cloudy. It can however be preserved for weeks, in well-stoppered containers. About 1 ml. of the fluid is sufficient for clearing 5 larvae at a time; and the same fluid can be used over again at least three times successively, provided care is taken to minimize introduction of water while transferring the larvae

composition:

Rectified spirit[2]	60 parts
Formalin	7 parts
Acetic acid	3 parts
Clove oil	40 parts

Cork the tube well and lay it aside until the larvae are well cleared and hardened. This would not take more than 15 to 30 minutes, depending on the size of the larvae; but there is no harm in leaving them longer in the fluid, even for days.

4. Transfer larvae into pure clove oil for 10–15 minutes during which time clearing would be complete.
5. Mount in canada balsam.

For Larval Skins

1. Stain skin in 0.5% aqueous eosin, for 20 minutes.
2. Wash well in two changes of distilled water.
3. Transfer to a cavity slide and, using a binocular microscope, cut the body wall along the mid-ventral line, with a pair of sharp microsdissection needles.
4. With a wide pipet transfer to a clean slide lightly smeared with Mayer's albumin.
5. Carefully draw off excess of water with filter paper, and spread out the skin on the slide.
6. Warm the slide above a flame until the skin is only just moist; *but do not let it dry.*
7. Cover the skin with a drop of clove oil and lay aside until clear.
8. Drain off the clove oil, replace it by canada balsam, and apply cover slip.

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from water to the fluid. This can be managed by using a curved needle for the purpose, instead of a pipet.

The rationale of the formula is as follows: Alcoholic clove oil is capable of clearing objects from 74% alcohol; the presence of acetic acid in addition to alcohol helps hold the clove oil in the solution. The fluid is actually a mixture of Bles' fixative (slightly modified) and clove oil. The mixture has been found to fix and to clear simultaneously, with a partial dehydrating action; the subsequent use of pure clove oil completes the dehydrating and clearing process.

[2]The alcohol content of the sample used was about 86% by volume. It proved easily available to the author and it costs only less than a tenth of absolute alcohol, including duty and gallonage charges.

ALDEHYDE REACTIONS IN TISSUES IN RELATION TO THE FEULGEN TECHNIC

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Received for publication June 23, 1950

ABSTRACT.—**The nature of the Schiff-positive radical responsible for the Feulgen reaction was studied by several standard tests for aldehyde. Oxidative, amine, alkali, and catalytic reactions for aldehydes were used. An aldehyde reaction, similar histologically to the Feulgen, could not be produced by the oxidative and amine technics. However, by means of alkali and catalytic methods, it was possible to block the Feulgen-positive tissue radicals ordinarily released by acid hydrolysis. A second hydrolysis of the tissues (after the blocking reactions) restored most of their original Feulgen-positive characteristics.**

The Schiff (1866) test for aldehydes was adapted to histological use by Feulgen and Rossenbeck (1924), Feulgen and Voit (1924 a, b, c), in the course of their studies on nucleic acids. After noting that acid-hydrolyzed thymonucleic acid became Schiff-positive, they subjected tissue sections to acid hydrolysis and then treated them with Schiff's reagent. They considered the resulting violet coloration of the nuclei as a specific demonstration of the cellular site of thymonucleic acid. Subsequent experimentation has to a great extent confirmed this theory and although a few dissenting papers have appeared, the Feulgen reaction is now generally accepted as specific for thymonucleic acid. Stowell (1946) summarized the various arguments concerning this specificity and concluded that if the reaction is performed properly and evaluated correctly it is specific. More recently, Dodson (1946), Di Stafano (1948), and Ely and Ross (1949) have come to similar conclusions.

Most of the past studies of the Feulgen reaction have been performed to determine the nature of the reacting *compound* within the tissues. Little attention has been paid to the actual reacting *radical* within the nucleic acid complex since it has been generally accepted as an aldehyde. The present experiments were performed to study this radical or radicals without regard to its parent compound.

Aldehydes are exceedingly active compounds (Karrer, 1950) and will react with a great variety of reagents including themselves. Consequently, if aldehyde radicals are produced by acid hydrolysis of thymonucleic acid, it should be possible to demonstrate them histo-

[1]Publication No. 548.

logically by some means other than the Feulgen reaction. The reactions which appeared to give the best promise of success were (1) metallic oxidative reactions, (2) reactions with primary amines, (3) alkali rearrangements, and (4) rearrangements in the presence of alkoxide catalysts.

The experimental procedure was divided into two parts: (1) Oxidative and amine reactions were used in an effort to demonstrate union between tissue aldehydes and reagents which had been substituted for Schiff's reagent. (2) The aldehyde rearrangement reactions were used as tests for the presence of aldehyde radicals by blocking subsequent reaction with Schiff's reagent. In each case, the experimental sections were checked against (1) sections which had been routinely stained by the slide or block Feulgen methods, (2) sections of tissue subjected to acid hydrolysis but not stained, (3) sections which were not hydrolyzed but treated with the test solution, and (4) sections which had received no treatment whatsoever. The procedures are listed in Table 1.

Two methods of hydrolysis were used, the slide technic of de Tomasi (1936) and the block method (Lhotka and Davenport, 1947, 1949). The slide technic depends upon hydrolysis of mounted sections in 1*N* HCl for five minutes at 60°C. The block method utilizes the hydrolytic action of a special fixative consisting of 2.5% sulfosalicylic acid in half-saturated aqueous picric acid for 24 to 36 hours at room temperature.

Aldehydes are known to be oxidized readily (Karrer, 1950) by a great variety of compounds, including the salts of heavy metals. Ammoniacal solutions of silver nitrate in the presence of aldehydes are reduced to the metallic form, and cuprous oxide can similarly be precipitated from alkaline solutions of cupric sulfate such as Fehling's and Benedict's solutions. A similar reaction is seen with bismuth in Nylander's solution. These solutions were substituted, under varying conditions of time, temperature, and concentration, for Schiff's reagent. In an attempt to facilitate histological examination, sections treated with Fehling's solution were treated also with ammonium sulfide in an effort to obtain the black copper sulfide. In no case were we able to demonstrate a specific aldehyde reaction of the Feulgen type.

The reactions of aldehydes with primary amines (Johnson *et al.*, 1946), such as hydroxylamine to form aldoximes, hydrazine to form hydrazones, and semicarbazide to form semicarbazones, have long been important in the detection of aldehydes. Behrens (1903) and Dakin (1908) recommended their use, and more recently, Bennett (1940) used a hydrazone in his study of the adrenal gland. The crystalline compounds formed by these reactions are obtainable easily, are fairly insoluble, and in many cases colored. This is particularly

true with such phenyl derivatives of hydrazine as phenylhydrazine, 2,4,dinitrophenylhydrazine, and p–nitrophenylhydrazine.

The reaction between aldehydes and primary amine compounds is believed to take place in two stages (Karrer, 1950), with an initial reaction between the amine and carbonyl group of the aldehyde, followed by a spontaneous splitting out of water:

$$R\text{–}CHO + R\text{–}NH_2 \rightarrow R\text{–}CHOH\text{–}NH\text{–}R \rightarrow R\text{–}CH{=}N\text{–}R + HOH.$$

Tissues which had been hydrolyzed both in the block and on the slide were treated with all of the above reagents under varying conditions of time, temperature, and concentration. Here, again, we were unable to demonstrate a sharp specific reaction in any way similar to that obtained with Schiff's reagent.

In a further attempt to demonstrate a union between tissue aldehydes released by hydrolysis and primary amines, blocks and sections which had been treated with p–nitrophenylhydrazine were placed in cold aqueous alkali (5*N* NaOH) in an effort to demonstrate the characteristic violet color of the Janovsky reaction (Johnson, 1950; Janovsky and Erb, 1886; Janovsky, 1891). P–nitrophenylhydrazones give excellent Janovsky reactions, and if this particular compound existed as a result of an aldehyde-amine reaction, it should have been possible to demonstrate it. However, only diffuse brownish coloration characteristic of the reaction between base and excess p–nitrophenylhydrazine remaining in the tissues occurred.

When aldehydes are treated with alkali (Adams et al., 1944) a dismutation occurs with the formation of an alcohol and a carboxylic acid:

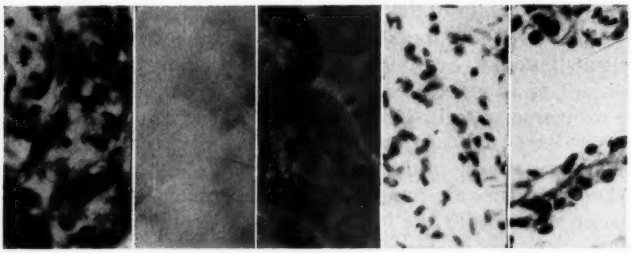
$$2\ R\text{–}CHO + NaOH \rightarrow R\text{–}CH_2OH + RCO_2Na$$

This rearrangement of aldehydes in the presence of alkali was observed first by Cannizzaro and now bears his name. If formaldehyde is introduced into the principal aldehyde solution to expedite the reaction, the resulting reaction is called a crossed Cannizzaro. We used this latter variation since we were dealing presumably with tissue-bound aldehyde radicals which had limited motion and could not be expected to react freely with each other. This experiment was performed only on blocks of tissue, since it was impossible to submit sections on slides to action of strong alkali without destroying them. Blocks, however, held up well for periods as long as four hours. The blocks, hydrolyzed by fixation in picro-sulfosalicylic acid mixture, were placed in a solution of 50% KOH in 50% alcohol (Adams et al., 1944) for periods ranging up to four hours. After removal from the solution, they were washed until no more alkali could be removed and then processed as shown in Table 1, B. This procedure definitely prevented the Feulgen reaction. If the material was rehydrolyzed by immersion for 24 hours in the picro-sulfosalicylic acid mixture, following the Cannizzaro reaction, it was possible again to demonstrate the presence of Schiff positive material in cell nuclei.

The reduction of an aldehyde to a primary alcohol through the medium of aluminum alkoxides (Adams et al., 1944) was first noted independently by Verley and Meervein in 1925. The following year Ponndorf demonstrated that a more selective reaction could be obtained by the use of isopropyl alcohol and aluminum isopropoxide in place of ethyl alcohol and aluminum ethoxide. The reduction of the aldehyde (Gilman, 1943; Adams et al., 1944) occurs at the expense of the alcoholic solvent, which is oxidized into an aldehyde. This aldehyde can then be removed from the solution with a stream of dry nitrogen, allowing the reaction to continue to completion:

$$R\text{–}CHO + CH_3CH_2OH \xrightarrow{Al(OC_2H)_3} R\text{–}CH_2OH + CH_3CHO\uparrow$$

If the aldehyde formed by the reaction is not removed, or another aldehyde is added to the initial solution, a rearrangement known as the Tishenko reaction may occur (Gilman, 1943). In this case two



Explanation of Fig. 1–5. Magnification × 125.

(Numbered from left to right)

FIG. 1. — Picro-Feulgen block stain of cat kidney.

FIG. 2. — Picro-Feulgen block stain of cat kidney following the Meervein-Verley-Ponndorf reaction. The absence of staining is characteristic.

FIG. 3. — Picro-Feulgen block stain of cat kidney after rehydrolysis of tissue which had been submitted to the Meervein-Verley-Ponndorf reaction. The nuclei are somewhat swollen but show Feulgen-positive material.

FIG. 4. — Cat lung fixed directly from the living animal with the picro-sulfosalicylic acid mixture and stained by the block technic.

FIG. 5. — Lung from same animal fixed in same manner five minutes after death and stained by the block Feulgen technic.

molecules of aldehyde are converted into an ester by means of the alkoxide catalyst:

$$2\ R\text{–}CHO \xrightarrow{AL\ (OC_2H_5)_3} R\text{–}CO_2CH_2\text{–}R$$

To obtain the Meervein-Verley-Ponndorf reaction, hydrolyzed blocks were refluxed from 2 to 4 hours in isoproyl alcohol (Adams et al., 1944) to which an excess of aluminum isopropoxide had been added. The Tishenko reaction was conducted in a similar manner

except for the addition of a sufficient amount of formaldehyde to furnish an adequate excess for ester formation. Following refluxing, the blocks were washed until all traces of alcohol disappeared and then processed as shown in Table 1, B.

Both technics were successful in blocking the Feulgen reaction, although the Meervein-Verley-Ponndorf method gave the better results. In both cases we were able to demonstrate the presence of Schiff positive material in the nuclei after rehydrolysis of the refluxed blocks. The blocking effect of the Meervein-Verley-Ponndorf reaction is shown in Fig. 1 and 2. The effect of rehydrolysis and reappearance of Feulgen positive material is shown in Fig. 3.

TABLE 1. SCHEDULES OF EXPERIMENTS.

Tissue Sections or Blocks	Hydrolysis	Oxidative Reagent or Primary Amine	Schiff's Reagent	Remarks
A.				
Experimental tissues	Yes	Yes	No	Negative in all cases
Control 1 (Routine Feulgen)	Yes	No	Yes	Regular nuclear staining
Control 2	Yes	No	No	Negative
Control 3	No	Yes	No	Negative
Control 4	No	No	No	Negative

Tissue Blocks	Hydrolysis	Alkali or Alkoxide Rearrangement	Rehydrolysis	Schiff's Reagent	Remarks
B.					
Experimental blocks	Yes	Yes	No	Yes	Feulgen reaction blocked
Control: Feulgen stain	Yes	No	No	Yes	Nuclear staining
Experimental blocks after rehydrolysis	Yes	Yes	Yes	Yes	Nuclear staining

It is difficult to come to definite conclusions regarding the results of our experiments. Failure to demonstrate the presence of aldehydes with such specific tests as the oxidative and amine reactions raises the question of the presence of aldehyde radicals as the cause of the Feulgen reaction. This doubt is not completely removed by our successes in the blocking reactions.

The possibility must be considered that the reacting radical is a ketone rather than an aldehyde. Many ketones show a positive reaction with Schiff's reagent and the blocking reactions are essentially characteristic of all carbonyls. However, ketones show a strong affinity for amines (Johnson et al., 1946) duplicating the aldehydes in this

respect. The failure to demonstrate an amine-carbonyl union greatly weakens such a premise.

The ability of certain nitrogen-containing compounds, especially those containing double bonds (Ex.: pyridine) to recolor Schiff's reagent is well known. It is conceivable that nitrogen-containing radicals might be made active, during acid hydrolysis of thymonucleic acid from the purine and pyrimidine rings, and that such radicals might be able to effect recoloration. Reactions through nitrogenous radicals, however, cannot explain the success of the blocking experiments, because such radicals are not affected by aldehyde condensations (Karrer, 1950; Gilman, 1943).

Another aspect not usually considered is that the Feulgen reaction may be a more complex mechanism than is generally believed and involves such factors as resonance (Pauling, 1940) and electronic bondings (Gilman, 1943). If this were the case, steric configurations which fit Schiff's reagent, but not other reagents for aldehydes, may be involved.

A puzzling circumstance related to the Feulgen reaction is concerned with post-mortem changes in tissues. When tissues are fixed from the living state by picro-sulfosalicylic acid fixative, the Feulgen reaction is weak. It would appear that the sudden precipitation of tissue proteins and the immediate initiation of acid hydrolysis prevents certain autolytic changes necessary for an optimum reaction. In spite of adequate hydrolysis, a sufficient number of reacting radicals apparently are not released. The same weakness of reaction can be shown if acetic sublimate fixative is substituted for picro-sulfosalicylic acid mixture and the tissues then processed by the slide technic. A similar but less marked effect can be seen if an attempt is made to fix and stain tissue at the same time in one solution (Lhotka and Davenport, 1947). The weakness of the reaction compared with the normal stain is shown in Fig. 4 and 5. This sign of nuclear change at the cessation of life appears to indicate that thymonucleic acid is held in a complex series of bondings, and that it must undergo some autolytic changes before it will give a maximum Feulgen reaction.

Before the mechanism of the Feulgen reaction is completely understood, the aldehyde assumed to be present should be demonstrated by other reactions in addition to that given by Schiff's reagent.

ACKNOWLEDGMENT

This research was supported by a grant from the Biological Stain Commission.

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DETECTION OF RADIO-ELEMENTS IN HISTOLOGICAL SLIDES BY COATING WITH STRIPPING EMULSION —THE "STRIP-COATING" TECHNIC

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ABSTRACT.—**The "strip-coating" technic offers some improvement of the published methods for the autographic detection of radio-elements in sections by stripping films. Reproducible results are easily obtained with Ilford Half Tone Stripping Plate Emulsion. The method does not lend itself readily to the preparation of the large number of slides usually required for biological research.**

The method of "autography" (radioautography, autoradiography, or histoautoradiography) became of practical use for solving histological problems when technics were devised to obtain an intimate

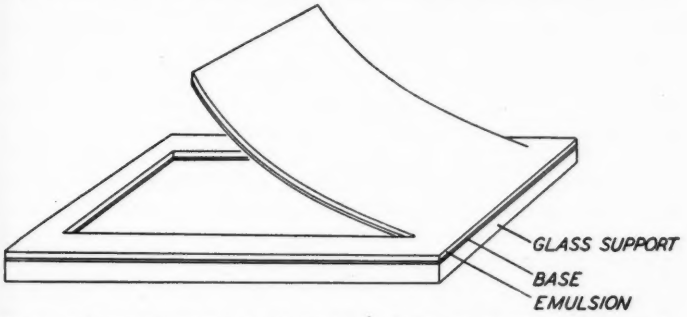

FIG. 1. — Schematic drawing of a stripping emulsion partly stripped from its glass support. Note that the emulsion is not separated from its base during stripping.

contact between a radioactive histological section and a photographic emulsion. This intimate contact was achieved by the "coating method" in which the section is coated with a melted photographic emulsion (Bélanger and Leblond, 1946; Leblond, *et al.*, 1948). This unity of emulsion and specimen produced a high resolution which allowed for a more precise localization of radio-elements in small areas of tissue (Gross, *et al.*, 1950).

Recently, emulsions that could easily be stripped from their base and then placed at the surface of the section were introduced by Pelc (1947) and Boyd and Williams (1948). In our experience, these stripping emulsions tended to be displaced in the course of processing, so that there was not a perfect juxtaposition of radioactivity sites and photographic reactions. The same difficulty was encountered by other workers, as may be seen from the inspection of published pho-

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tographs (Andresen, *et al*, 1950). An attempt was made, therefore, to find out the cause of these shifts or displacements; and, as a result, a modified technic for coating sections with stripping emulsion was devised. With this technic displacements were avoided and results as satisfactory as with melted emulsions were obtained.

The stripping emulsion used in the tests was from Ilford Half Tone Stripping plates. The method was later attempted with NTB_1 stripping film and NTB_2 stripping plate developed by the Eastman Kodak Company.

The Ilford stripping emulsion is mounted on a double backing: (1) a loosely attached glass support, and (2) a thin, pliable base (gelatin) intimately attached to the emulsion (Fig. 1). The emulsion with its base was easily peeled off from the glass support and was soaked in water for 2 minutes prior to being applied on the histological section in which radioactivity was to be detected (Pelc, 1947; Gross, *et al*, 1950). Under these conditions, there was always a more or less definite displacement of the autographic image in relation to the sites of radioactivity in the section. The existence of such displacements was discovered by comparison with preparations employing the coating method (Leblond, *et al*, 1948), in which the autographic images always perfectly coincide with the structures causing them. Since in the course of exposure the latent image was formed in close contact with its source, the displacement must have occurred later, that is, during development or fixation, and presumably because of the swelling of the emulsion which occurred during these operations.

The displacements observed in the preparations were of two types: (a) generalized, with all reactions displaced at the same distance from their source, and (b) localized, with only a few reactive areas showing displacement. To prevent the *generalized* displacement of the emulsion, an attempt was made to keep the emulsion tightly attached to the slide. After various trials, the method that best accomplished this consisted of using large pieces of emulsion strips, and folding the overlapping edges over the sides of the glass slide (Fig. 2).

The *localized* displacements were on the other hand attributed to uneven soaking of the emulsion possibly causing folding and uneven swellings. To prevent them, the duration of soaking was increased from 2 to 5 minutes and a wetting agent, Dupanol C (lauryl sulfate), was added to the water in the hope of obtaining a faster and more even penetration of the water into the emulsion.

Experimental

Prevention of Emulsion Displacement. The thyroid gland of a rat given 25μc of radioiodine one day before sacrifice was sectioned at 5μ, mounted on 3″ × 1″ glass slides that had been covered with the usual thin layer of egg albumin, stained in hematoxylin-eosin, and coated with 1% celloidin. Four sets of slides were used to test the

amount of displacement in autographs under different conditions. The emulsion with its base was soaked in a 1% solution of dupanol in distilled water. Preliminary experiments had indicated that dupanol has no effect on the sensitivity of the emulsion.

(a) The first group of slides was covered with strip emulsion that had soaked for *two* minutes in the dupanol-water solution. The emulsion strip edges were folded under the slides on *three* sides (one narrow and two long sides), as shown in Fig. 2.

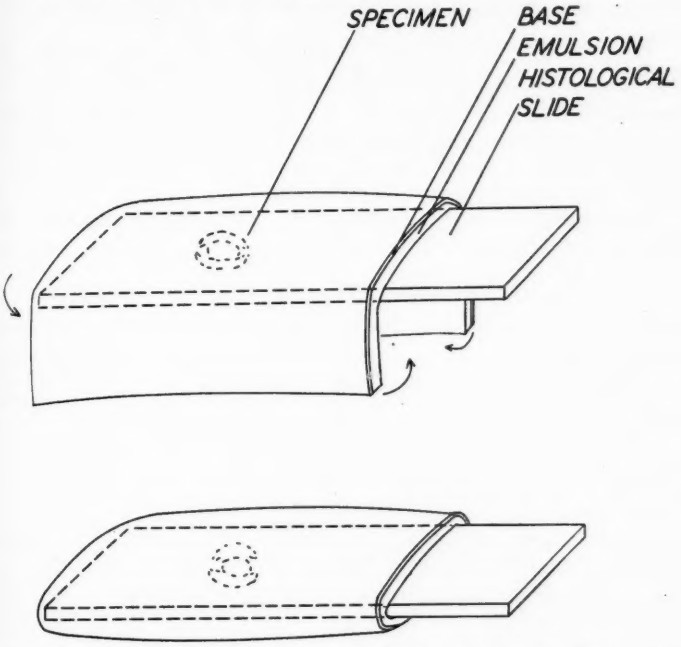

FIG. 2. – Schematic drawings of the "coating" of a histological slide with stripping emulsion.

In the *upper drawing* the histological slide is slipped underneath the emulsion so that three flaps of emulsion hang from three sides of the slide. The arrows indicate where these flaps will be folded under the slide to give the finished preparation as seen in the *lower drawing*. Note that the emulsion is most proximal to the slide whereas the base is distal to it.

(b) The second group was covered with strip emulsion that had soaked for *five* minutes in the dupanol solution. The emulsion strip edges were again folded under the slides on *three* sides.

(c) and (d) The third and fourth groups were similar in soaking time to groups one and two respectively, but only *two* side edges of the emulsion strip were folded (along the two long sides of the slides).

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The results seen in Table 1 and Fig. 3 and 4 indicated that an interval of two minutes was not sufficient soaking time for Ilford Strip emulsion and that folding on two sides only did not give consistent results.

The method adopted consisted of soaking Ilford Strip emulsion for *five* minutes in a dupanol-distilled water solution and applying it so that *three* flaps could be folded under to meet on the undersurface of the slide. Any type of displacement was thus prevented. This method proved satisfactory in a number of subsequent experiments.

Other Technical Studies. Preliminary experiments had indicated that a better resolution was obtained when the *outer free surface of the emulsion (Ilford) was placed in direct contact* with the section. In this way the base faced outwards rather than in contact with the section. It may be noted that the base of the Ilford stripping emulsion is permeable to developer and fixer.

It was found indispensable to dry the preparations completely before *storing them for exposure.* The slides were placed on a carrier

TABLE 1. EFFECT OF SOAKING TIME OF STRIP EMULSION ON AMOUNT OF DISPLACEMENT.

Number of slides	Time in distilled water and dupanol	Number of edges folded under	Number of slides in which displacements occurred
6	2 minutes	3	3
6	2 minutes	2	6
6	5 minutes	3	0
6	5 minutes	2	2

which allowed the two long edges of the slide to remain free, while the narrow edges fitted on a rest made of thin supports in such a way that only a minute portion on the back of one narrow edge of the slide was in contact with the carrier. Furthermore, the slide was kept horizontal when drying. Drying was carried out in a desiccator containing calcium chloride. The dry preparation fitted easily in an ordinary slide box, which could be placed in a light-tight container for exposure. Storage of Ilford Strip covered preparations during exposure was in the deep-freeze, after it was determined that the low temperatures had no adverse effect on the autographs.

The *development and fixation* of the strip autograph slides were carried out as usual (Gross, *et al.* 1950) except that a prolonged fixation was required, presumably because of the high silver bromide concentration of the emulsion. Fifteen minutes was the time taken for the emulsion to clear at 18–20°C.; and, therefore, 25 minutes were deemed necessary for complete fixation. Washing in running water required a minimum of thirty minutes, preferably 1 to 2 hours. The slides were kept horizontal throughout development and fixation.

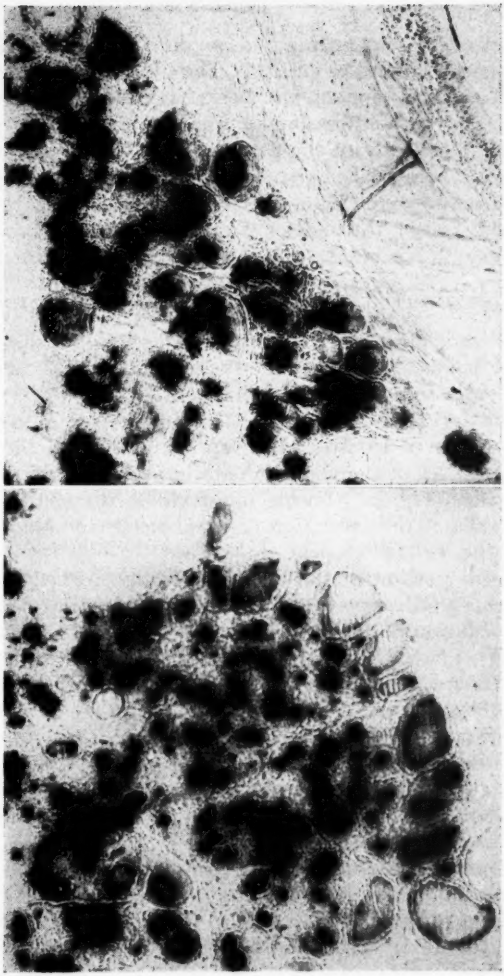

FIG. 3 and FIG. 4. — Strip coated autographs of sections of a thyroid gland from a rat injected subcutaneously with I^{131} and sacrificed 24 hours after injection. In Fig. 3 (top) the autograph was prepared by soaking Ilford Half-Tone Stripping Plate emulsion for *2 minutes* in distilled water and dupanol. *Two* of the emulsion strip edges were folded under the slide. In Fig. 4 (bottom) the autograph was prepared by soaking the emulsion strip for *5 minutes* and folding the edges of the strip under *three sides* of the slide.

(The autographic reaction is predominately in the colloid except at the periphery of the gland in Fig. 4 where the reaction is in the apex of the cells.)

In Fig. 3 note that the autographic reaction does not correspond exactly with the histological details of the colloid indicating a *displaced* autograph. There is no displacement in Fig. 4 where the reactions of the colloid coincide exactly with the location of the colloid.

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The method of *dehydrating and mounting* was that described in other technics (Leblond, *et al,* 1948). The slides were then cleaned by wiping off the emulsion on the back. However, if the excess was trimmed away before the preparation was completely mounted, there was a possibility of slight displacement or even total lifting of the emulsion strip. The balsam mounted autograph was, therefore, allowed to dry overnight, after which time the emulsion lying outside the coverslip and on the back of the slide was trimmed away with a razor blade.

Technical Details of the "Strip-Coating" Method

1. The histological sections are mounted on a glass slide and may be stained or unstained. They are then taken through alcohol-ether + 1%-celloidin solution and dried.

2. In the darkroom (Wratten 1 Safelight) the emulsion strip with its base is removed from its glass support by cutting the emulsion along three edges of the glass and slowly peeling it off to one inch from the edge (Fig. 1). Blowing moist air on the emulsion during stripping reduces the formation of static electricity and thereby reduces fogging.

3. The cut portion of the emulsion is then turned away, page-like, from its glass support, and is placed in a shallow dish containing a 1% solution of dupanol in distilled water at 18–20°C. for five minutes. This is done in such a way that the side of the strip which formerly adhered to the glass faces upward. It may be found convenient either to rest the intact glass support on the edge of the dish during this soaking, or to break the glass so that only one inch remains as support for the emulsion, which can then be rested on the edge of the dish. Finally, however, it may be more desirable to cut pieces of required size from the strip and to place them with or without support in the soaking solution.

4. The histological slide with the section facing upward is slipped underneath the emulsion, care being taken not to trap air bubbles. Then, partly covered with emulsion, the slide is lifted out of the water. The emulsion is cut away from its glass support and trimmed to a little larger size than that of the slide (Fig. 2, above) so that the edges may be folded under three sides of the slide to insure adherence (Fig. 2, below).

5. The slides are next placed in a desiccator until they appear completely dry. This takes from 15 minutes to two hours. They are stored in light-tight slide boxes which are themselves kept in light-tight jars containing a drying agent. The jars are transferred to a deep freeze for the duration of exposure.

6. Development is carried out in D–72 for one and a half minutes. Fixation is in acid fixer for 25 to 30 minutes. Washing is in running water for 30 to 60 minutes.

7. The washed preparations are dehydrated and cleared through three minute changes of 95% alcohol, one change of absolute alcohol, one change of alcohol-xylene (1:1), three changes of xylene and left overnight in a 1% solution of Canada balsam in xylene. The preparations are mounted in balsam, covered with a coverslip and dried overnight. The emulsion folded over the edges is then cleaned off.

In the *Eastman Kodak NTB_1 stripping film,* the support consists of celluloid instead of glass, but the method of peeling the emulsion strip remains the same. However, the base of this emulsion, unlike that of Ilford stripping emulsion, is waterproof and does not allow the penetration of developer and fixer. Therefore, the base side of the emulsion must be placed against the specimen and thus the access of developer and fixer to the emulsion is made possible, but some loss in resolution results. The NTB_2 stripping plate emulsion on the other hand is very similar to the Ilford plates and can be handled in the same way. These emulsions have the advantage of being thinner than the Ilford emulsion, but they are rather more difficult to handle.

It was furthermore found that when NTB_1 film and NTB_2 plates were used, the extreme cold temperature of the deep freeze caused the strip to peel away from the slide, at times ripping the tissue section off the slides. These preparations were, therefore, exposed in an electric refrigerator at temperatures not lower than 0°C. Even at these temperatures, they occasionally came off the slide.

Recently, autoradiographic plates have been made available by the British Kodak Company. The described method was successfully applied to these plates. Although they strip more easily than do the Ilford plates, they appear to be less sensitive; but since the thickness of this Kodak emulsion is only 5 μ whereas that of the Ilford plates is about 40 μ, and the grain sizes are approximately the same, a better resolution may be expected with the Kodak emulsion (Gross *et al).* While this paper was in press, Berriman, Hertz, and Stevens (1950) described a method for using this emulsion for radioautography in which displacement was prevented. This method consists of coating the slides with gelatin before mounting the sections on it. We have had no experience with this method.

Discussion

The "strip-coating" technic requires less apparatus than the fluid coating technic described previously, since it eliminates the need for a constant temperature water bath and a leveling table. In addition, this technic has the advantage of a uniform thickness of the emulsion coating. This, however, will not be achieved unless the emulsion is thoroughly soaked (5 minutes in water at 18–20°C.) and is not stretched during processing.

The results obtained using Ilford stripping film were more satisfactory than those obtained with NTB_1 stripping film and NTB_2

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stripping plate. These latter emulsions are difficult to handle since they tear easily during application to the glass slide. Furthermore, storage at temperatures below 0°C. tends to lift the strip coating from the glass slide often pulling the histological section with it. However, the NTB_2 emulsion has the advantage of a far greater sensitivity than the Ilford; and, therefore, must be used for low activity preparations.

In a comparison study made between the strip coating and liquid coating methods using Ilford half tone stripping film, it was found that there was no essential difference in the quality of the results obtained (emulsion thicknesses being equal). For routine autographic work, however, the liquid coating method was found to be the method of choice since as many as 6 dozen autographs can be coated in one hour, whereas the number of sections coated with strip emulsion per hour is less than 2 dozen.

The strip coating technic as described may be used, especially where a minimum amount of apparatus is available and when only a small number of preparations is required.

Acknowledgment

This work was supported by a grant from the Banting Research Foundation.

The help of Dr. C. P. Leblond who suggested this method and advised me throughout the work is acknowledged, as is the assistance of Mr. H. Torunski in the preparation of the histological material and of Miss Cimon for the drawings.

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PROPYLENE AND ETHYLENE GLYCOL AS SOLVENTS FOR SUDAN IV AND SUDAN BLACK B[1]

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Received for publication July 1, 1950

ABSTRACT.—**Propylene or ethylene glycol is recommended as a solvent for Sudan IV and Sudan black B to replace the commonly used alcohol-acetone mixtures for general lipid staining in tissue sections. Either glycol is used as a dehydrating agent, dye solvent, and differentiating solution. They offer the advantages of a stable solution, inert with respect to solubilities of lipid material in it, and excellent control of differentiation without loss of dye from lipid particles. Sections remain pliable and are not shrunken by the glycols. Counterstains may be used after staining with Sudan IV but are generally not necessary after staining with Sudan black B. With the use of propylene glycol as a solvent, Sudan IV appears to equal the staining ability of Sudan black B as regards the type of lipid material detected, and the choice of dye to be used would depend on the color contrast desired.**

Propylene glycol and ethylene glycol, used as solvents for the azo and diazo "Sudan" dyes, have been found superior to alcohol-acetone mixtures as formerly used for the detection of lipid substances in tissue sections. The glycols have overcome most of the disadvantages of other vehicles and fulfilled all requirements of an efficient solvent, namely that it (a) should not extract lipid substances from tissue sections, (b) should dissolve sufficient quantities of oil-soluble dyes to yield brilliant staining results, (c) should be easily, rapidly, and economically prepared and stable in stock solution over long periods of time without deterioration, evaporation, or precipitation. In numerous preparations, no difference in variety of lipid substances stained or in the intensity of staining has been found between Sudan IV and Sudan black B dissolved in propylene glycol, although Sudan black B has been described as revealing a greater range of lipids than Sudan III or IV (Lison and Dagnelie, 1935; Sheehan, 1939; Bailiff and Kimbrough, 1942; McManus, 1945; Discombe, 1946; Sheehan and Storey, 1947; Bunting, Wislocki, and Dempsey, 1948; Sheehan and Whitwell, 1949).

Excellent discussions of the theory of lipid staining as well as the chemical formulae of the oil soluble dyes (other than Sudan black B which was then of unknown chemical structure) and their char-

[1]Supported by a grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

acteristics are available elsewhere (Conn, 1946; Lillie, 1948). The structurally similar "Sudan" dyes (II, III, IV, and oil red O) are very weakly acidic or neutral, almost insoluble in water, and incapable of forming salts; they are slightly soluble in alcohol and very soluble in lipids and lipid-solvents such as acetone. The solubility of the dyes in 70% alcohol can be increased sufficiently by the addition of acetone, as in the usual preparations, to yield fair staining results in tissue sections. However, such a mixture has certain disadvantages. The 70% alcohol has been known to dissolve out small portions of lipid material from sections (Govan, 1944; Lillie, 1948), and acetone rapidly extracts neutral fats and fatty acids from tissue (Mallory, 1938; Lillie, 1948). Since sections are usually passed through three successive alcoholic solutions (dehydration, staining, and "rinsing"), there exists a question of how much lipid material or dye has been extracted by the solvents. In preparation, the dyes are usually dissolved to saturation point in an alcohol-acetone mixture, and the amount dissolved decreases with reduction in the concentration of alcohol and the evaporation of acetone. The dye concentration in such solutions is uniform for short periods of time only. Frequent filtering is necessary to avoid deposits of precipitate on the section. Prolonged "rinsing" to remove such dye precipitate extracts a certain amount of absorbed dye from the lipid particles and may, in addition, extract lipid from the section. The usual short "rinsing" time is difficult to control from section to section.

Different solvents have been reported by other investigators. Lillie and Ashburn (1943) described isopropyl alcohol as a vehicle for the Sudan dyes, oil red O, and oil red 4B (Lillie and Ashburn, 1943; Lillie, 1944). However, except in the case of oil red O and oil red 4B, the dye solutions were unstable and deteriorated upon standing. A watery medium of 1% gelatin has been used by Govan (1944). The solubility of Sudan black B in several organic acids has been tested by Cohen (1949), who was using it for a different purpose, however. Hartman (1940) found ethylene glycol superior to 70% alcohol, 50% dioxane and water, and 50% acetone and water as a solvent for Sudan black B for staining certain bacteria in wet preparations. Glycol did not evaporate in wet mounts and retained excellent staining ability for many months without apparent deterioration. Alcohol and acetone were inferior because of their hygroscopic properties resulting in progressive reduction of dye solubility. Dioxane was noxious and undesirable to handle for long periods of time.

In the present investigation, the recommended procedure was evolved after numerous tests of effective dye concentration, time of staining, time of differentiation, concentration of differentiating solution, and tests of lipid solubility in the glycols. With all types of tissue, the results have been consistently superior to the former methods of handling these dyes. In addition, Sudan IV dissolved in pro-

pylene glycol stains myelin sheaths, mitochondria, and other lipids as well as Sudan black B. The matter of color contrast desired would seem to indicate which of the two dyes should be used in any particular tissue, rather than which dye stains the greater variety of lipid. In the following tests, sections of kidneys that contained large amounts of lipid material were most frequently used; they were obtained from autopsy material and from experimental animals. Frozen sections were selected for uniformity of thickness in comparing results of staining in each test.

Dye Concentration in Glycols

Propylene glycol, ethylene glycol, and dihydroxy-diethyl-ether were tried in addition to some of the above mentioned solvents. Both propylene and ethylene glycol have similar properties. Propylene glycol dissolved a greater amount of both Sudan IV and Sudan black B than does ethylene glycol, generally giving more brilliant and rapid staining although no difference was apparent in the variety of lipid substances detected. When saturated solutions of either dye prepared in both propylene and ethylene glycol were compared spectrophotometrically, the shape of absorption curves was similar but the propylene glycol solution had a greater optical density at all wave lengths, indicating an increased solubility of dye in propylene glycol. Both Sudan IV and Sudan black B approached saturation point, at room temperature, at 0.5 g. per 100 ml. of propylene glycol. Dihydroxy-diethyl-ether solutions stained well, but differentiating solutions of this substance rather rapidly removed some of the dye from lipid particles. Increasing concentrations of Sudan black B, from 0.4% to saturation point, showed little visible difference in staining ability. Below the level of 0.3%, however, staining was progressively diminished. Spot tests of the solutions were compared for color intensity, and although there was a gradual increase in coloration, there was very little difference between the 0.3% and 0.7% solutions. When Sudan IV was used, there was a distinct increase in brilliance of stained lipid substances as the concentration increased to saturation point.

Staining Time

Sections were stained for intervals of 3, 5, 10, 20 and 60 minutes and then differentiated for a constant interval of 5 minutes in 85% glycol and water. The time of staining did not show a significant difference between the type of solvent used, either propylene or ethylene glycol. Sections stained for 3 minutes showed slight irregularity and diminished intensity of coloration. Sections stained longer than 5 minutes showed no appreciable increase in color intensity of lipid substances, although there was a gradual increase in tinting of cytoplasmic background with both Sudan IV and Sudan black B.

The cytoplasmic background staining is due to staining of tiny particles, chiefly mitochondria, but in no instance did this confuse the identification of the brightly stained lipid substances or particles.

Differentiating Time

Sections of kidney were stained for a constant period of 10 minutes and then differentiated in 85% propylene or ethylene glycol and water for periods of 1, 5, 10, 20 and 60 minutes. At 1 minute the differentiation was good but not all of the excess dye was removed. At 5 minutes the section was completely clear of excess dye and no change was seen in the intensity of lipid coloration. At 10 minutes there was only very slight loss of lipid staining when ethylene glycol was used, but no loss with propylene glycol after this interval. Differentiating times of 20 and 60 minutes gradually extracted the dye from the lipids. Tinting of the cytoplasm, at times certainly due to the staining of mitochondria, decreased with increasing time in the differentiating solutions until it was completely lost after 10–20 minutes. A differentiating time of 2–3 minutes appeared ample for either dye in either solvent. The latitude in differentiating time for good results had a decided advantage over the conventional "rapid rinsing" in 70% alcohol.

Concentration of Differentiating Solution

Sections were stained for 10 minutes and then differentiated for 3 minutes in graded concentrations of propylene or ethylene glycol, from 100% to 50%, and in distilled water. Differentiation in 100% glycol resulted in marked and sometimes complete loss of stain from lipid substances. This was also evident, but to a lesser degree, in the 95% solution. At concentrations of 90% or lower, there was an increased retention of dye in the cytoplasm. There was no difference in the differentiation in 50% glycol and distilled water.

Solubility of Lipid Material in the Solvents

Formalin-fixed frozen sections of many organs, including brain, liver, kidney, subcutaneous tissue, and adrenals were tested for loss of sudanophilic material after being exposed for varying lengths of time in distilled water, glycerine, 100% ethylene glycol, 100% propylene glycol, 70% alcohol, and absolute acetone. Sections exposed over 1 hour were kept in a refrigerator to avoid decomposition. Control sections of each type of tissue were stained immediately by the outlined method, and at the end of two months, their microscopic appearance had not changed. As might be expected, there was no loss of staining of mitochondria, myelin, or other lipid substances from sections kept in distilled water. There was no loss of sudanophilic material from sections kept in glycerine, ethylene glycol, or

propylene glycol. Sections kept in acetone showed immediate (1 hour) loss of lipid from adipose tissue, liver cells, Kupffer cells, and renal tubular epithelium; mitochondria and myelin staining was preserved for 2 months. There was no effect of 70% alcohol on the staining of myelin or mitochondria, but there was some loss of lipid particles in Kupffer cells after 1 hour, complete in 48 hours. Alcohol removed some of the neutral fat droplets in the renal tubules after 48 hours and approximately 80% of it in 2 months. Alcohol, as used in the conventional procedure, is capable of extracting lipid from certain small deposits even though the exposure is brief. It is true, however, that this is not significant when dealing with large deposits of lipid.

Method

The following procedure for preparation of the dye solution and its use in staining tissue sections has been employed in this laboratory for nearly 2 years as a general lipid stain.

A. Preparation of Dye Solution. Seven tenths of a gram of Sudan IV or Sudan black B is dissolved in 100 ml. of pure propylene or ethylene glycol by heating to 100–110°C. and thoroughly stirring for a few minutes. Care should be taken not to exceed 110°C., since a useless gelatinous suspension will result. Filtering the hot solution through Whatman No. 2 paper removes most of the excess dye and undissolved impurities. After cooling to room temperature, it is filtered again through a fritted-glass filter of medium porosity with the aid of suction. As an alternative the solution may be filtered through paper for the second time, but the procedure is long, due to the viscosity of the glycols. The glycol solvents should be obtained in as nearly pure form as possible, since an appreciable water content (30–40%) will prevent much of the dye going into solution.

B. Staining Procedure.

(1) Cut frozen sections to desired thickness and wash in water for 2–5 minutes, or longer, to remove excess formalin.
(2) Dehydrate the sections in pure propylene or ethylene glycol for 3–5 minutes. Whichever solvent is used for the dye, that solvent is used both for dehydration of sections and differentiation after staining. Occasional movement of the sections in solutions is desirable because of the viscosity of the glycols.
(3) Transfer sections to the staining solutions for 5–7 minutes. Agitate the sections occasionally.
(4) Transfer sections to a solution of 85% glycol and water and move back and forth gently for 2–3 minutes.
(5) Transfer sections to distilled water for 3–5 minutes and then to a slide; drain excess water and mount in hot Kaiser's glycerine-gelatin.

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COUNTING ERTHROCYTES AND LEUCOCYTES IN FOWL BLOOD

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Received for publication July 8, 1950

Simple diluting fluids such as that recommended by Loissons are satisfactory for avian red cell count; this method, however, gives insufficient differentiation to enable accurate white cell counts to be made. To overcome this limitation, Olson (1935) adopted the method of Wiseman in which phloxine is used to stain the red cells and acidophil granulocytes. The leucocyte count is calculated by multiplying the acidophil count by a factor obtained by making a differential count in a stained blood smear.

In order to simplify procedure the substitution of phloxine by a more specific stain was considered. It has been found that this two-stage technic increases the possibility of experimental error. Giemsa, Wright's, Lieshmann and Mann's stains were investigated: Mann's stain was found to be too variable in its reactions; Wright's and Lieshmann's (purchased in methanol solution) when diluted enough for alcohol interference to be lost, gave insufficient intensity of staining; Giemsa stain proved to be sufficiently specific and possessed the virtue of being free from the disadvantages associated with these other stains.

TECHNIC

A. The diluting fluid: Solution 1: Sodium chloride 0.66 g.
Sodium citrate 1.11 g.
Neutral formalin 5 ml.
Distilled water 95 ml.

Solution 2: Giemsa solution (B.D.H.); 1 ml. of solution to be added to 9 ml. solution 1.

Important detatils are: (a) The diluting fluid should be made up fresh daily. (b) The solution should have a pale violet tinge. If the color does not appear then, 1% sodium carbonate must be added drop by drop until it does appear (Carleton, 1947). (c) The solution should be filtered through a Whatman No. 1 filter paper into shallow capsules ready for use.

B. The count: Blood obtained directly from a prick in the wing vein, is sucked up to the 0.5 mark on a red cell diluting pipette. After the outside of the pipette has been cleaned of blood with filter paper, diluting fluid is sucked up to the 101.0 mark giving a final dilution of 1/200. The pipette is rotated to distribute the cells evenly and a

broad elastic band stretched lengthwise over the pipette to prevent loss of fluid. The pipette is then placed in the refrigerator for four hours. For the actual count, the Neubauer haemocytometer is most satisfactory, as a total of 9 mm. may be counted in the case of the white cells. The white cells counted in this area when multiplied by 2,000/9, are equal to the total white cells per millimeter. The red cells are counted in 80 of the smallest squares. The figure obtained is multiplied by 10,000 which gives the total red cell count per millimeter. The reader is referred to Whitby and Britton (1948) for details on the use of the haemocytometer and the precautions that must be taken to avoid inaccuracy. With experience the cell types are readily recognized with the aid of the 4 mm. objective.

The *red cells* are easy to recognize; they are oval in outline and have a centrally placed nucleus. Red cell nuclei without cytoplasm might be confused with lymphocytes, except for the fact that they are a much lighter blue in color.

Platelets are the smallest blood cells. They are oval in shape, often with one extremity larger than the other. The nucleus is centrally placed, and blue-purple in color. One or two red granules may be seen at one or both poles of the cell. The platelets frequently occur as clumps and are thus difficult to count.

Lymphocytes are the most numerous of white cells. They differ widely in shape and size. The smaller lymphocytes possess a round densely staining nucleus which may be slightly indented at one side; a pale blue staining cytoplasm forms a narrow crescent bordering one side of the nucleus. Larger lymphocytes possess more cytoplasm.

The *monocytes* are difficult to distinguish from large lymphocytes. Since they are both counted in the white cell count, determining the exact nature of white cell type is not important.

The *granulocytes* can be recognized by the presence of specifically staining granules and are counted with the lymphocytes and monocytes in the total white cell count.

Discussion

The leukosis complex, Jungherr, Durant and Lee (1941), accounts for an extremely high proportion of deaths in the domestic fowl (Coles, 1950). The serious incidence of this disease in the poultry industry and its close relationship to leucaemia in man, Engelbredth-Holm (1942), has meant that a great deal of fundamental research is carried out on problems associated with this disease. Thus the importance of an accurate but simple method for enumerating white and red cells in the blood of fowls is obvious and it was to overcome the difficulties associated with previous technics that this method was designed.

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NOTES ON TECHNIC

Treadle Power For Rotary Microtomes

For many years we have mounted our rotary microtomes on discarded bodies of treadle sewing machines and have found this a useful and convenient procedure. Since visitors to our laboratories have admired this simple device, I thought it ought to be communicated to a larger audience before all the old fashioned treadle machine bodies become extinct.

As shown in the photograph, a plain wooden top on which the microtome rests is provided. Through this top two slots about 2 inches by ¾ inch are cut for the leather belt which passes over

the microtome balance wheel and down around the machine wheel actuated by the treadle. To insure a constant and proper position for the instrument with reference to the belt slots, two pieces of moulding are nailed to the table top fitting along the back and right rear of the microtome. One must add about an extra foot of belting to an ordinary leather sewing machine belt to make it long enough.

One generally starts the balance wheel by hand and switches to foot power when the paraffin ribbon is coming off nicely. This frees both hands for taking care of the ribbon. With a little experience foot control becomes very accurate and one can cut at a slow even

rate with great precision. We have found the control much superior to that possible with an electric motor provided with foot pedal rheostat.

The table top of the machine serves well to hold the section trays. The drawers in the cabinet are convenient for holding accessories for cutting. The whole unit can be moved from room to room with ease and safety, but the most important advantage lies in an accurately controlled cutting movement which frees both hands of the operator to handle the paraffin ribbons. – S. I. KORNHAUSER, *Department of Anatomy, University of Louisville, School of Medicine.*

A MODIFICATION OF DELAFIELD'S HEMATOXYLIN FOR RELATIVELY THICK SPECIMENS

It is sometimes desirable to stain total mounts of relatively thick specimens (1 to 2 mm.) with a general nuclear stain. Due to the thickness of the mount, however, it is very difficult to obtain good differentiation using the Delafield technic. The following method has been found very convenient in preparing total mounts of *Actinosphaerium*, a multinucleate heliozoan, which is approximately one millimeter in diameter.

After fixation and washing, the tissue is transferred through increasing concentrations of alcohol to 70% alcohol and is then stained in strong Delafield's hematoxylin for 5 to 15 minutes. The entire process is best carried out in a Columbia culture dish. One solution is substituted for another by means of medicine droppers. After overstaining in hematoxylin, the tissue is placed in glycerine to which 0.5% of hydrochloric acid has been added. The glycerine renders the tissue partially transparent and the progress of destaining may be clearly observed through the microscope. When the proper intensity of stain has been reached (usually the nuclei will appear red while the cytoplasm is pale pink), several drops of a saturated solution of lithium carbonate (in water or 70% alcohol) are added. The nuclei will then appear deep purple. At this point the tissue may be washed in water or it may be transferred directly to 70% alcohol. If desired, a counterstain such as eosin, erythrosin, or orange G may be applied in the usual way. The tissue is then transferred through absolute alcohol into xylol and mounted in balsam or in gum Damar.

The use of acid glycerine rather than acid alcohol for differentiation of Delafield's hematoxylin permits better control of the process since the tissue is rendered relatively transparent in glycerine and allows continuous observation of the destaining, whereas the specimen is sometimes completely opaque in alcoholic solution. – GERARD M. LEHRER, *Department of Biology, The City College of New York.*

Oxalic Acid as a Destaining Agent for the Ferric Chloride Tannic Acid Technic

In recent years, one of the most useful and satisfying microtechnical methods has been the ferric chloride, tannic acid staining technic. It has proven to be a most useful tool for the research worker and the neophyte alike.

Frequently, when beginners or others are using this staining method either on meristematic or mature tissues, the sections will be overstained. In order to recover valuable sections which might otherwise be discarded or reduced in value, a suitable destaining agent has been needed.

The writer has observed that if one employs an old household remedy for removing rust spots from fabric, the overstained sections may be salvaged. The worker may make up aqueous solutions of oxalic acid in varying concentrations which will either completely remove the stain or give better differentiation. For general work in this laboratory a 0.1% solution has been found to be most satisfactory. The technician merely needs to take the overstained sections from water, place them upon the stage of the microscope and add a drop or two of the oxalic acid solution. When the destaining has proceeded far enough, transfer immediately to water, thus stopping the action of the oxalic acid. — Roy Milton Chatters, *Department of Botany & Plant Pathology, Oklahoma A. & M. College, Stillwater, Okla.*

Higher Polyethylene Glycols as a Water Soluble Matrix for Sectioning Fresh or Fixed Plant Tissues

Sectioning fresh plant tissue in a matrix of water-soluble wax offers a method of obtaining uniform sections in which the enzyme and substrate reactive systems are little disturbed. For example, the freezing of sections of meristems destroys the reaction in which 2, 3, 5–triphenyltetrazolium chloride is reduced to the red formazan. However, if "Carbowax" 4000 is used as an embedding medium, with the freezing microtome, the reduction of the tetrazols to formazans is not disturbed. The writer has used this method on ovules of peppers and observed the tetrazolium reaction to be clearly defined in "Carbowax" embedded material; whereas in frozen sections the reaction is blocked. This observation has been verified by Roberts[1] in a general survey of tetrazolium reactions by meristems.

"Carbowax" has also been found to be useful in embedding fixed

[1]Roberts, L. W. 1950. A survey of the causal factors responsible for the reduction of 2,3,5–triphenyltetrazolium chloride by plant meristems. Thesis. University of Mo. (Subm. for publ.)

tissues for preparation of sections for the electron microscope.[2] Tissues were embedded in "Carbowax" directly from water. Blocks were then sectioned into small fractions of a μ (0.1–0.2μ). "Carbowax 4000 which melts near 50° C. was the type of wax used by Anderson and Hance.[2] The general characteristics of "Carbowax" 4000 and other polyethylene glycols may be found in a publication by Carbide and Carbon Chemicals Corporation.[3]

Fresh sections of uniform thickness may be prepared quickly and easily by the following method. The freezing microtome was used as the sectioning instrument; however, carbon dioxide was not necessarily employed. The "Carbowax" 4000, obtained from the Carbide and Carbon Chemicals Corporation, was placed in a small beaker and melted on a hot plate. The temperature was maintained only slightly above the melting point. This avoided injury to tissue and undue delay in hardening of the wax. Enough wax was poured onto the stage of the microtome to coat at least two-thirds of its surface. The material to be sectioned was then arranged as desired as the wax hardened. Wax was added to cover the material and then allowed to harden. Hardening was frequently hastened by use of a small amount of gas but never enough to cause chilling of the tissue. The surface to be cut was slightly softened by applying water with a moist brush. Brushing with this moist brush between each cut also prevented flaking of the wax and greatly facilitated sectioning. Sections were then washed in water to remove any particles of wax clinging to them.

Some woody and tough herbaceous materials may be sectioned with greater ease in a frozen mixture of "Carbowax" and water. The percentage of Carbowax may be varied to fit the particular needs of the material. Fifty percent solutions produced a mixture that held material substantially longer than water alone. — STANLEY R. MCLANE, JR., *Department of Horticulture, University of Missouri.*

[2]ANDERSON, T. F., and HANCE, R. T. 1942. A microtome sectioning technique for electron microscopy illustrated with sections of striated muscle. Proc. Soc. Exp. Biol. and Med., **51**, 148–52.

[3]CARBIDE and CARBON CHEMICALS CORPORATION. 1946. "Carbowax" compounds and polyethylene glycols.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

GATENBY, J. BRONTE, and BEAMS, H. W. (editors). **Lee's Microtomist's Vade-mecum. A Handbook of the Methods of Animal and Plant Microscopic Technique.** *11th Ed.* 5½ x 8½ inches. *Churchill, London; Blakiston, Philadelphia.* $8.50.

It has been 13 years since the 10th edition of this book was published, and the new edition is welcome, even though the Vade-mecum is no longer the only treatise covering this field. This book has now become a collaboration, like the one reviewed just below; in the 10th edition the editors had ten collaborators, in the 11th, sixteen (the death of one of whom during the course of preparation was responsible for a considerable part of the delay in getting out the new edition). In the 10th edition Dr. Gatenby's associate editor was Dr. Painter; in the 11th it is another American, Dr. Beams.

A critical review of such a work as this cannot be written off-hand; obviously continued use under actual laboratory conditions is needed before it can be properly evaluated in comparison with earlier editions. It is clear, however, by merely glancing through the pages that the editors and their collaborators have made a painstaking effort to bring the subject matter thoroughly up to date. It contains much revised material, and also entirely new or completely rewritten chapters: e.g., one on Laboratory Cultivation of Invertebrata, by Libbie Hyman; on Fatty Substances, by R. Whitehead and W. W. Kay; on Microchemistry of Substances other than Fats, by G. Gomori (whose authorship has to be inferred from the preface, as it unfortunately is not indicated where the chapter occurs). To include the new material, space requirements forced the omission of certain old sections, among which is the chapter on dye chemistry and staining; this latter omission, as explained by the preface, is not through ignorance of the work of the Stain Commission (in fact the junior editor is a Commission member) or from lack of appreciation, but rather because the subject is felt to be treated adequately in readily available Commission publications — such as the journal in which this review appears.—*H. J. Conn.*

JONES, RUTH McCLUNG (editor). **McClung's Handbook of Microscopical Technique.** *3rd Ed.* 6 x 9 inches. *Paul B. Hoeber, New York.* 1950.

This well known collaboration ran through two editions during the life of its original editor, Dr. C. E. McClung; the second edition was exhausted a little before his death and he asked the present editor, his daughter, to undertake the revision. The revision has been eagerly awaited, and its publication is now greatly welcomed by those interested in this field. It is, in fact, no mere revision in the usual sense, but has been very largely rewritten, and it is easy on glancing it through to understand some of the reasons for delay in finishing it. It includes the work of 35 collaborators, some new to this edition, others contributors to one or both of the earlier two. The various contributions have been carefully harmonized, so as to make a more consistent whole than was previously the case.

Much new material has been added, some of it lengthening chapters previously included (e.g. those on fluorescence microscopy, bone and cartilage, embryology), the rest included in new chapters with new authors: on electron microscopy; on

microradiography; on polarization microscopy. The last mentioned subject is one that is not readily available to biologists elsewhere.

Including all this new matter made it necessary for the editor to exclude some subjects previously included. With much reluctance, it was finally decided to exclude all methods of strictly botanical or bacteriological application. The book, therefore, is now primarily one in animal microtechnic, although many of the methods described are of such general application as to give the book value to botanists as well as zoologists. This new edition, in fact, should prove useful in almost any biological laboratory where microscopic methods are employed.— *H. J. Conn.*

LANGERON, M. **Précis de Microscopie.** *7th Ed.* 5 x 8 inches. 1430 pages. *Masson, Paris.* 1949.

Langeron's book is not one of the conventional treatises on histologic technic, but it attempts to inform the reader about all phases of biological and medical work, in which the microscope is used. It begins with an excellent discussion of the use of the microscope and its accessories, in which the phase-contrast microscope and the electron-microscope (which according to the author is only an "enlarger") are not forgotten. Apart from sections on tissue processing, cutting and staining (in which much space is devoted to the methods used in the laboratory of P. Masson) there are chapters on microscopic technic in protozoology, in the study of virus diseases, helminthology, entomology, hematology, the examination of sputa and serious effusions, special histologic technics, cytology, histochemistry, bacteriology, botany, mycology and forensic medicine. The author has made every effort to bring the book up to date and in this he has succeeded fairly well. In the sections on protozoology and bacteriology the composition of several important culture media are also given. In the chapters devoted to staining methods a number of technics evolved in France during the war, are included. The section on "Histochimie et Analyse Chromatique" is very complete.

It is obvious that in a book of this scope not every section can be equally complete. It is, however, an excellent guide for a small laboratory and indispensable for workers in the tropics, who do not have a large technical library at their disposal, and who do not want to be burdened with many books on scientific expeditions, and yet wish to have a reliable guide for their technical work.— *Ph. H. Hartz. Curacao*

MICROSCOPE AND OTHER APPARATUS

DRUMMOND, D. G., editor, and others. **The practice of electron microscopy.** *J. Roy Micr. Soc.*, **70**, *1–141.* 1950.

The Electron Microscopy Group of the Institute of Physics, Cambridge University, has prepared, with Dr. D. G. Drummond, as editor, an extensive treatise on the practice of electron microscopy. The present article is a revision of the typescript edition published by the Institute of Physics in 1948. There are ten other contributing editors whose specialties and contributions cannot be mentioned in such a short review. There are ten chapters listed below with relevant comments. Four appendices with twenty-two plates complete this most comprehensive treatise.

Chapter I deals briefly with general optical considerations. Basic technics are included in Chapter II where the familiar procedures are critically analyzed and presented. Examination of small particles constitutes Chapter III. Special preparations are outlined. Determination of size and shape of particles are admirably described. In Chapter IV, air borne particles receive the following discussion: (1) sedimentation and impingement; (2) thermal precipitation; (3) electrostatic precipitation. Thin film specimens and surface replicas are given adequate consideration in Chapter V.

Microörganisms are treated under the following headings in Chapter VI. (a) simple preparations; (b) methods to prevent distortion; (c) study of development of organisms; (d) methods of increasing transparency; (e) micro-location of specimens.

An excellent presentation of biological tissues is discussed in Chapter VII. Technics of extraction in solution by the volatile salt method as well as the spraying method are clear.

Chapter VIII comprehensively presents six special technics that are associated with electron microscopy, such as, (1) freeze-drying; (2) heavy metal staining; (3) section cutting; (4) shadow casting; (5) stereo-micrograms and six special devices.

No treatise on electron microscopy is complete unless calibrations of magnifications and resolving power are presented. Chapter IX adequately does just that. The final chapter describes up-to-date photographic technics applicable to electron microscopy.

The four appendices discuss the following: (a) practical illuminating aperature and resolving limit; (b) the stability required in control circuits; (c) magnification and distortion at the projector lens; (d) effects of tilting the incident beam.

For one who is familiar with special fields of electron microscopy, this treatise offers an overall picture that permits one to appreciate the advances made in an adjoining area of investigation. An excellent list of references is given.

There are excellent books on the electron microscope. This article is what its title indicates: The *Practice* of electron microscopy. To do this, experts are necessary to describe their specialties. We have here an outstanding collection of detailed accounts that meet all the requirements for fine use of such a versatile tool.—*Paul G. Roofe.*

PHOTOMICROGRAPHY

SAYLOR, CHARLES PROFFER, BRICE, ARTHUR T., and ZERNIKE, F. **Color phase-contrast microscopy: requirements and applications.** *J Opt. Soc. Amer.*, **40,** 329–34. 1950.

For the use of color phase contrast in microscopy, which was first discussed in the spring of 1948, several difficult requirements must be met for the attainment of maximum brilliance and usefulness. The system itself is one in which a controlled chromatism of the phase plate is sought instead of the customary control of phase difference at one wave-length only. The thickness and dispersion of the phase ring in relation to the general aperture can be such that zero phase contrast will occur at one or more wave-lengths of transition in color sensation. If the phase contrast is made high but opposite in several zones of relatively pure color sensation, striking and beautiful microscopical images are formed with objects of low inherent contrast. The greatest advantage of the system lies not in the beauty of the images, but in the clear distinction which is afforded between effects caused by scattering of general absorption and those caused by small differences of refractive index or thickness. Two photomicrographs in color of protozoa and onion root tip are shown.—*G. C. Crossmon.*

MICROTECHNIC IN GENERAL

MACDONALD, A. M., COBB, JACK, SOLOMON, A. K., and STEINBERG, D. **Stripping film technics for radioautographs.** *Proc. Soc. Exp. Biol. and Med.*, 72, 117–21. 1949.

Eastman Type M stripping film was cemented, emulsion side up, to mounted sections with a 1% solution of celloidin in methyl alcohol. The stripped transparent supporting base was laid over the emulsion and a second slide placed on top of the whole. The edges of the two slides were then bound together with

Scotch tape to form a "sandwich" of the section and sensitized film. Each preparation was wrapped in opaque paper and placed in the dark under a pressure of 8 lb./sq. inch for an exposure period of about 15 days. After exposure, the slides were separated by cutting the binding tape along the edges of the slides with a razor blade. Unwinding the tape caused static discharges and fogging. Five minutes development of the film in Kodak D–19, 15 sec. in 1% acetic acid solution and 2 min. in Kodak F–5 fixing solution constituted the processing of the radioautograph. To stain the section, the film and section (which loosen during development) were carefully stripped from the slide, washed, dehydrated through alcohol, and recemented *section side up* to another slide with Kodalith Stripping Film Cement or a 1% solution of celloidin in ether-alcohol. Staining, dehydrating and covering were then done in the routine manner. The authors recommend the use of Eastman No-Screen x-ray film for making test exposures to determine the proper exposure for Type M stripping film. Since the x-ray film is much more sensitive than the Type M, trial exposures with sections from the same block of tissue to be used with Type M can be made and the exposure time for the final preparation estimated. A table, showing relative densities obtained from C^{14}, Ca^{45}, I^{131}, P^{32}, and Zn^{65}, is given. The data indicate that Type M film would require 10–40 times the exposure needed for No-Screen x-ray film.—*H. A. Davenport.*

ANIMAL MICROTECHNIC

GORIROSSI, F., and DEGIUSTI, D. L. **A modified gold chloride impregnation method for trematode nerve tissue.** *Trans. Amer. Micr. Soc.*, **69,** *183–5.* 1950.

Methods developed for mammalian nerves did not stain the nervous system of Haematoloechus, a lung fluke. The following modifications gave satisfactory preparations: Kill the nematode, which has been washed free from mucus and debris with saline solution and straightened out on a bit of filter paper, with a small amount of 1% formic acid, 40°C, 5 min.; drop the nematode and filter paper into cool 1% formic acid for 15–20 min.; wash in distilled water 5–10 min.; dehydrate through a graded series of isopropyl alcohol; clear in oil of cedarwood; embed in paraffin; section at 7μ; mount using albumin fixative; remove paraffin; hydrate; place in 1% formic acid 1–5 min.; rinse in distilled water 2–3 min.; drain; place in 0.2% gold chloride at 39.5°C. for 8–16 hr. (the longer the time in formic acid the shorter the time in gold chloride) keeping the solution carefully covered; rinse with distilled water; reduce the gold chloride by immersing sections in 1% formic acid at 39.5°C. 4–16 hr. (shorter time for longer impregnation); wash in distilled water 10 min.; dehydrate rapidly through isopropyl alcohol series, clear in xylene; and mount in balsam or clarite.—*Virgene Kavanagh.*

MUELLER, J. D. **Some observations on the structure of Hydra, with particular reference to the muscular system.** *Trans. Amer. Micr. Soc.*, **69,** *133–47.* 1950.

A technic which demonstrates clearly the muscle fibers of hydras is the following: Drop about 12 hydras into 10 ml. Flemming's solution, strong formula, cooled to near 0°C. and standing in an ice bath, from a pipette with a minimum amount of water; replace the fixative with a fresh chilled solution and place in the ice bath in a refrigerator for 12–72 hr. (optimum 24); wash in running tap water 24 hr.; dehydrate; embed in paraffin; section at 4μ or less and mount; remove paraffin; hydrate; mordant in 4% aqueous iron alum, 1 hr.; wash 30 min. in running tap water; place in 1% aqueous acid fuchsin, 30 min.; pass directly to Lugol's iodine solution, 2 min.; rinse in distilled water; dehydrate through graded alcohols; from 95% alcohol pass to 0.5% solution light green SF yellowish in 95% alcohol, 15 min.; differentiate in 95% alcohol; pass through carbol-xylene to xylene; mount in damar. A little orange G added to the light green solution gives a richer color. The stain does not give uniform results, but a successful one

has green cytoplasm, red or green cytoplasmic granules, red nuclei, bluish to green mesoglea, green support structures and bright red muscle fibers.—*Virgene Kavanagh*

PLANT MICROTECHNIC

COLEMAN, L. C. **Nuclear conditions in normal stem tissue of *Vicia Faba*.** *Canad. J. Res., Sec. C.*, **28,** 382–91. 1950.

To obtain better understanding of the nature of tumors induced in stems of *Vicia faba* in inoculation with *Agrobacterium rubi,* it was considered necessary to know whether normal stem tissue is wholly diploid or partly polyploid. Mitoses were induced by treating with indole-3-acetic acid and the chromosomes were studied by the following method: Cut treated section including the upper node and the internode immediately below; remove cortex on the side opposite to the treatment; fix the remaining tissue in 3 parts alcohol and 1 part acetic acid; wash in water; hydrolyze in normal hydrochloric acid 15 min. at 60°C.; leave in leuco-basic fuchsin 30 min. to 24 hr.; wash thoroughly in sulfur dioxide water; carefully separate material to be studied (cortex and pith) from the remaining tissue; squash small pieces on the slide in 50% acetic acid. To make permanent: leave slides overnight in dioxane; separate cover slip from slide; mount in dioxane balsam, clarite or euparal. A very considerable degree of polyploidy was found in both the pith and the cortex.—*H. P. Riley*

MICROÖRGANISMS

BUEDING, ERNEST, and KOLETSKY, SIMON. **Content and distribution of glycogen in *Schistosoma mansoni*.** *Proc. Soc. Exp. Biol. and Med.*, **73,** 594–6. 1950.

Material for study was obtained by injecting mice intraperitoneally with 160 cercariae of *S. mansoni* and removing the flukes from the mesenteric and portal veins after 6–23 weeks. Glycogen in both males and females was determined chemically by the method of Good, Kramer and Somogyi, and also stained with carmine. The worms were stained after fixation in alcoholic Bouin's fluid for 18 hr., washing through 3 changes of absolute isopropyl alcohol and 2 changes of xylene, 30 min. each. Author does not state that the specimens were embedded; presumably they were stained whole, first in alum hematoxylin without bluing, followed by Best's differentiating fluid (methyl alcohol, 10 ml.; 50% ethyl alcohol, 5 ml.) The staining solution consisted of carmine, 0.4 g., potassium carbonate, 0.2 g., potassium chloride 1.0 g., and distilled water 12 ml. The ingredients are mixed, brought to a boil, and allowed to remain over the flame until the solution has turned a deep red color. After cooling, 4 ml. of concentrated ammonia water is added. This stock solution is diluted with 15 ml. of concentrated ammonia water and 15 ml. of methyl alcohol before use. The glycogen content of the male worms was greater by about 5 to 1 than that in the females, and it tended to increase with age in the males but not in the females. Good correlation between the chemical determination and the intensity of staining was observed.—*H. A. Davenport.*

CASSEL, W. A. **The use of perchloric acid in bacterial cytology.** *J. Bact.*, **59,** 185–9. 1950.

The use of cold perchloric acid to remove pentose nucleic acids avoids the possibility of heat artifacts. The method, as employed for *Bacillus cereus,* is as follows: Prepare a saline spore suspension from a culture on potato extract agar; inoculate the surface of a brain heart infusion agar plate and incubate for 3 hr.; cut an agar block from the plate and spread on a slide; fix in osmium tetroxide vapor for 1½ min. or in Schaudinn's fluid (63 ml. 6% $HgCl_2$, 32 ml. 95% ethyl

alcohol, and 5 ml. glacial acetic acid) for 20 min.; treat with normal perchloric acid at 4°C. for 30–40 hr.; rinse in tap water; stain with 0.1% aqueous basic fuchsin (Coleman & Bell 480345). These preparations are clearer than those prepared with normal HCl at 60°C. for 7 min.; HCl does not remove any appreciable amounts of pentose nucleic acid in 40 hr. at 4°C. At 70°C. the action of perchloric acid is very rapid, giving excellent pictures in 15–30 sec., fading after 1½ min. and disappearing after 7 min. treatment; the action of HCl is slower, with the chromatinic bodies appearing in darkly-staining cytoplasm at 30 sec., clearly visible at 2 min. and still distinct at 7 min.—*V. Kavanagh.*

FOURNELLE, H. J., and MACY, H. **A comparative study of presumptive media for the coliform group.** *Amer. J. Pub. Health,* **40,** 934–42. 1950.

Added to standard lactose broth (without bile), brilliant green was slightly more inhibitory than crystal violet in preventing gas production by coliform cultures in the presence of milk. The effective bacteriostatic concentrations of these dyes (1: 10,000 or greater) were considerably higher than the concentrations used in presumptive media (brilliant green in bile broth: 1/75,188; crystal violet in violet red bile agar: 1/500,000) specified in *Standard Methods for the Examination of Dairy Products,* 9th edition. These and other data showed that coliform tests, for evaluating the productivity of presumptive media, should be set up on a quantitative basis, and that mixed, rather than pure cultures, should be used as testing material.—*M. W. Jennison.*

LURIA, S. E., and HUMAN, M. L. **Chromatin staining of bacteria during bacteriophage infection.** *J. Bact.,* **59,** *551–60.* 1950.

To study the action of bacteriophage on *Escherichia coli,* it was necessary to use a liquid fixative to prevent premature lysis. The following method was used: To a measured sample of the culture, add twice its volume of a formalin-dichromate fixative of such strength as to make the concentration of the mixture equal to 3.7% formaldehyde and 2% $K_2Cr_2O_7$; let it stand 20 min.; centrifuge; wash; centrifuge; suspend in water; spread on water agar and make impression slides; place immediately in 70% alcohol until ready to hydrolyze with 1*N* HCl at 60°C. and stain with Giemsa solution (Nat. Aniline) 1:10.—*Virgene Kavanagh.*

PENNINGTON, D. **A reaction of bacterial cells with formaldehyde.** *J. Bact.,* **59,** *617–23.* 1950.

Bacterial cells fixed with commercial formalin (5% formaldehyde, pH 3.0–3.8) and stained with a basic dye such as thionin are uniformly well-stained; those fixed with neutral formalin (pH 7.0) varied in staining reaction. About half of the cells in young cultures, but relatively few of those in older cultures were stained. To obtain the effect, the living cells had to react with neutral formaldehyde. Drying, previous fixation with fixatives below pH 5, picric acid, OsO_4 vapor, or alcohol prevented the differential staining. Thioglycolate, hydrogen peroxide, potassium ferricyanide, ninhydrin, and nitrosourea had no apparent effect on subsequent fixation and staining.—*Virgene Kavanagh.*

SMITH, A. G. **Electron and light microscopic studies of bacterial nuclei. II. An improved staining technique for the nuclear chromatin of bacterial cells.** *J. Bact.,* **59,** *575–87.* 1950.

Impression-film preparations fixed in OsO_4 vapor were hydrolyzed with 1*N* HCl at 60°C. for appropriate periods, which varied not only with the species but also with culture medium and age of culture. They were then washed in water, mordanted in 1% formaldehyde 2–4 min., washed, stained in 0.3% aqueous basic fuchsin 15–30 sec., washed, and mounted in tap water. For electron microscopy, a collodion membrane was overlaid on the nutrient agar and implanted with tiny drops of bacterial suspension from a capillary pipette. Suitable colonies were

subjected to the various steps of the cytological fixation and staining with a colony mounted for examination on etched copper screen at each step.—*Virgene Kavanagh.*

HISTOCHEMISTRY

BLACK-SCHAFFER, B. **The tinctorial demonstration of a glycoprotein in Whipple's disease.** *Proc. Soc. Biol. and Med.*, **72,** 225–7. 1949.

An isotropic refractile substance found in the macrophages of the lamina propria of human intestine was found to stain positively with the HIO_4–Schiff reaction. The substance was present in apparently undiminished amounts in tissue kept for 13 years in aqueous Kaiserling's solution. It was not stained by either osmic acid or Best's carmine, but was tinted by Mallory's mucicarmine. Thus the staining reactions indicate that it is a glycoprotein, and this is substantiated further by its insolubility in water. The author concludes that this material may be the key to the etiology of Whipple's disease, and that its absorption may be due to some defect in the function of the intestinal mucosa.—*H. A. Davenport.*

GOMORI, GEORGE. **Further studies on the histochemical specificity of phosphatases.** *Proc. Soc. Biol. and Med.*, **72,** 449–50. 1949.

Previous studies (Proc. Soc. Exp. Biol. and Med., **70,** 7, 1941) were extended by the use of 10 substrates tested by incubating with tissue sections at pH 5.0 and at pH 9.2. The substrates were: catechol– and hydroquinone monophosphates; glucose–6–phosphate, fructose–6–phosphate, phosphoglyceric acid; yeast adenylic acid (3–nucleotide), muscle adenylic acid (5–nucleotide); dimethyl– and diphenyl acid pyrophosphates, and casein. In the alkaline range, positive reactions were obtained with all substrates, except the pyrophosphates and casein, within 2 hr. Casein was negative for all tissues in both acid and alkaline reactions. Pyrophosphates gave a positive reaction after 24 hr. at pH 9.2, and a weak, variable one at pH 5.0. Strong reactions for 5–nucleotidase were noted in axons and cell nuclei, and most smooth muscle. The evidence strengthened the opinion that 5–nucleotidase is a specific enzyme, different from nonspecific alkaline phosphatase. In the acidic reaction, phenylpyrophosphate tended to show a predominantly nuclear pattern of activity.—*H. A. Davenport.*

GOMORI, G. **Sources of error in enzymatic histochemistry.** *J. Lab. & Clin. Med.*, **35,** 802–9. 1950.

Histological technics for detection of hydrolytic enzymes may have errors due to false localization caused by supersaturation of the incubating medium with products of hydrolysis or by too long incubation of slides at low pH values. Most false negatives and false positives can be avoided by careful procedures. Quantitative results should be based on comparison with known standards.—*John T. Myers.*

GOMORI, GEORGE. **Histochemical localization of true lipase.** *Proc. Soc. Exp. Biol. and Med.*, **72,** 697–700. 1949.

Water-soluble substrates of long chained fatty acid esters of polyglycols were tested for esterase action by various tissues from man, dog, cat, guinea pig, rabbit, rhesus monkey, rat, mouse and pigeon. The substrates included both saturated and unsaturated fatty acid esters. The unsaturated esters were hydrolyzed almost exclusively by the pancreatic type of esterase (true lipase) while saturated esters were attacked by esterases of both hepatic and pancreatic type. Kidney, positive in reaction to saturated substrates, was consistently negative to unsaturated. Tween 80, G–2144, G–9446N and G–7627DJ (oleates); G–6486 (ricinoleate) and G–9926T (linseed), all of Atlas Powder Co., Wilmington, Del.; Neutronyx R

(oleate), Onyx Oil and Chemical Co., Jersey City, N. J.; and Antarox B290 (ricinoleate), General Analine and Film Corp., New York City, were satisfactory unsaturated substrates. A possible clinical connection between the low iodine number of serum lipids, associated with cystic fibrosis of the pancreas of children and also in infantile exzema, may exist and may be conditioned by hypofunction of pancreatic lipase.—*H. A. Davenport.*

KING, THOMAS J., and NIGRELLI, ROSS F. **Glycerophosphatases in the normal and tumerous frog kidney.** *Proc. Soc. Exp. Biol. and Med.*, **72,** 373–6. 1949.

The reactions of normal and adenomatous frog kidneys were studied by means of a modified (Kabat and Furth, Amer. J. Path., **17,** 303, 1941) Gomori method for alkaline phosphatase, with sodium beta-glycerophosphate used as the substrate. The normal kidney showed a strong positive reaction in the proximal tubules and a positive but less strong reaction in the glomeruli. Tumor tissue was negative, and even the normal tubules in the region immediately adjacent to the tumor were negative to weak in reaction. The high concentration of alkaline phosphatase in the proximal tubules suggests that this enzyme may play a role in sugar absorption.—*H. A. Davenport.*

KURINCK, N. B. **The quantitative estimation of desoxyribose nucleic acid based on methyl green staining.** *Exp. Cell. Res.*, **1,** 151–9. 1950.

Highly polymerized DNA fixes one molecule of methyl green to each 10 atoms of phosphorus. Depolymerization of DNA (notably following heating at 60°C.) entails loss of ability to fix methyl green; depolymerized DNA stains with pyronin.—*J. Dufrenoy.*

SMITH, CHRISTIANA. **Glycogen in basophilic leucocytes in human blood smears.** *Proc. Soc. Exp. Biol. and Med.*, **72,** 209–10. 1949.

Smears stained with Wright's stain in absolute methyl alcohol for 3 min., but with dilution of the stain with water omitted, were observed for basophils, and the location of each cell of this type marked with an object marker. The smears were then subjected to the HIO_4–Schiff reaction. Controls were digested with saliva and digested and undigested ones compared for staining of glycogen. Staining of the basophils in undigested smears varied from rather large clear red granules through fine dust-like particles to an absence of reaction in a few cells. Controls were all negative. It was concluded that a positive reaction for glycogen in human basophilic leucocytes was demonstrated by the procedure employed.—*H. A. Davenport.*